ESL-TR-85-38

Anaerobic Degradation of C_1 and C_2 Chlorinated Hydrocarbons

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DECEMBER 1985

FINAL REPORT

JUNE 1984 - JUNE 1985

AFESC

ENGINEERING & SERVICES LABORATORY
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AD-A165005

REPORT DOCUMENTATION PAGE

1a REPORT SECURITY CLASSIFICATION: UNCLASSIFIED	1b RESTRICTIVE MARKINGS
2a SECURITY CLASSIFICATION AUTHORITY	3 DISTRIBUTION/AVAILABILITY OF REPORT: Approved for public release, distribution unlimited.
2b DECLASSIFICATION/DOWNGRADING SCHEDULE	
4 PERFORMING ORGANIZATION REPORT NUMBER(S): ESL-TR-85-38	5 MONITORING ORGANIZATION REPORT NUMBER(S)
6a NAME OF PERFORMING ORGANIZATION: Air Force Engineering and Services Center; 6b OFFICE SYMBOL (If applicable): RDVW	7a NAME OF MONITORING ORGANIZATION
6c ADDRESS (City, State and ZIP Code): Tyndall AFB FL 32403	7b ADDRESS (City, State and ZIP Code)
8a NAME OF FUNDING/SPONSORING ORGANIZATION: AF Office of Scientific Research; 8b OFFICE SYMBOL (If applicable)	9 PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER
8c ADDRESS (City, State and ZIP Code): Bolling AFB Wash. D.C. 20332	10 SOURCE OF FUNDING NOS: PROGRAM ELEMENT NO: 62601F; PROJECT NO: 1900; TASK NO: 70; WORK UNIT NO: 28
11 TITLE (Include Security Classification): Anaerobic Degradation of C_1 and C_2 Chlorinated Hydrocarbons	
12 PERSONAL AUTHOR(S): Gossett, James M., Dr., Cornell University, AF OSR/URRP	
13a TYPE OF REPORT: Final; 13b TIME COVERED: FROM June 84 TO June 85; 14 DATE OF REPORT (Yr, Mo, Day): December 1985; 15 PAGE COUNT: 153	
16 SUPPLEMENTARY NOTATION: Availability of this report is specified on reverse of front cover.	

17 COSATI CODES			18 SUBJECT TERMS (Continue on reverse if necessary and identify by block number)
FIELD	GROUP	SUB GR	Anaerobic Degradation, Hazardous Wastes, Biodegradation, Solvents, Biological Treatment, Microbial Metabolism
06	13		
11	11		

19 ABSTRACT (Continue on reverse if necessary and identify by block number)

This research investigated the potential for anaerobic, biological degradation of five chlorinated C_1 and C_2 hydrocarbons: tetrachloroethylene (PCE), trichloroethylene (TCE), 1,1,1-trichloroethane (1,1,1-TCA), chloroform (CF), and dichloromethane (DCM). Attempts were made to delineate the importance of microbial -- as opposed to purely chemical -- mechanisms of degradation; products formed; and pathways involved. The five compounds were studied separately in batch, anaerobic systems employing a diverse community of microflora from a municipal waste treatment plant. The added substrate concentrations ranged from 2-25 mg/L for the C_1 compounds, and from 1-2 mg/L for the C_2 compounds.

20 DISTRIBUTION/AVAILABILITY OF ABSTRACT	21 ABSTRACT SECURITY CLASSIFICATION	
UNCLASSIFIED/UNLIMITED ☒ SAME AS RPT ☐ DTIC USERS ☐	UNCLASSIFIED	
22a NAME OF RESPONSIBLE INDIVIDUAL: Albert N. Rhodes, 1Lt, USAF	22b TELEPHONE NUMBER (Include Area Code): (904) 283-2942	22c OFFICE SYMBOL: RDVW

DD FORM 1473, 83 APR EDITION OF 1 JAN 73 IS OBSOLETE

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SECURITY CLASSIFICATION OF THIS PA

Radiotracer studies were conducted with ^{14}C-chloroform and ^{14}C-dichloromethane to investigate their fate, as well as to delineate degradative pathways. Interpretation of data required knowledge of Henry's constant for each substrate and product. Therefore, Henry's constants were measured for 14 compounds over a temperature range from 10° to 35°C, and over a range of ionic strength from 0 to 1 M (KCl).

PCE was completely converted to TCE, but the product was not further degraded to any significant degree. Only trace levels of 1,2-dichloroethylene isomers and vinyl chloride were detected. Results suggest that the microbially mediated reductive dechlorination of PCE is an inducible process.

Anaerobic conversion of 1,1,1-trichloroethane did not require acclimation. A major portion of the compound (at least 40 percent) was reductively dechlorinated to 1,1-dichloroethane, which accumulated. The lack of an acclimation period suggests that 1,1,1-TCA is processed by a constituitive enzyme system (or at least one already functioning in this inoculum). In studies where 1,1,1-TCA was repeatedly restored to batch systems, the rate of its utilization remained unchanged.

Anaerobic, microbial utilization of chloroform (CF) proceeded without acclimation. Since CF is not a natural substrate, its metabolism may result from its resemblance to some other, commonly encountered substrate or metabolite. It is unclear whether the mediating microorganisms derive energy or other growth-related benefit from utilization of CF. The rate of utilization was proportional to the remaining CF concentration (to some positive exponent). Approximately 31 percent of the degraded CF was converted to dichloromethane (DCM), which accumulated for an extended period before exhibiting a slow decline. Far smaller quantities of chloromethane (CM) were also detected. Studies with ^{14}C-chloroform indicated that 32 to 44 percent of added CF was converted to CO_2, with insignificant evolution of CH_4.

Microbial degradation of DCM occurred readily after an acclimation period of variable length. Results from repeated additions of DCM to acclimated systems suggest that acclimation to this compound may be sustained indefinitely, so long as even a modest level of DCM is maintained in the system. However, the ability of a system to degrade DCM is rapidly lost in its absence; exposure to DCM-free conditions for as short a period as 4 hours resulted in a subsequent lag period of about 4 days. These results imply that DCM degradation serves a purpose for the mediating microorganisms, and that an inducible enzyme system is involved. Radiotracer studies demonstrated that approximately 86 to 92 percent of the carbon from DCM was converted to CO_2. No direct conversion of DCM to CH_4 was detected. On the other hand, results show a definite link between DCM utilization and methane production. This suggests that the electrons removed in the oxidation of DCM to CO_2 are used to reduce some other compound (likely CO_2) to CH_4.

CF inhibits utilization of DCM in a manner that is slowly reversible. Inhibition persists long after levels of CF in the solution are depleted. These findings have significance with respect to the potential use of a microbial system for renovation of a groundwater simultaneously contaminated by both CF and DCM; biological treatment would appear to be most efficient if a sequential scheme were employed -- with CF conversion accomplished to DCM and CO_2 in a first stage, followed by mineralization of DCM in the absence of CF in a second stage.

EXECUTIVE SUMMARY

A. PROGRAM OF STUDY

The purpose of this research was to investigate the potential for the anaerobic, biological degradation of five, common, chlorinated C_1 and C_2 hydrocarbons: tetrachloroethylene (PCE), trichloroethylene (TCE), 1,1,1-trichloroethane (1,1,1-TCA), chloroform (CF), and dichloromethane (DCM). Attempts were made to delineate the importance of microbial — as opposed to purely chemical — mechanisms of degradation; products formed; and pathways involved.

The five compounds were studied separately in batch, anaerobic digestion experiments employing a diverse population of anaerobic microflora from a municipal waste treatment plant. The concentrations of the added, volatile substrates -- as well as the occurrence of volatile intermediates and products — were routinely assayed using a headspace chromatographic technique. The added substrate concentrations ranged from 2-25 mg/L for the C_1 compounds, and from 1-2 mg/L for the C_2 compounds. Two types of control systems accompanied the viable, seeded systems: autoclaved-seed controls, and distilled-water controls. All incubated systems consisted of 158.8 mL serum bottles with 100 mL liquid volumes, sealed with Teflon^{TM}-lined rubber septa and aluminum crimp caps. All incubations were carried out at constant temperature (35°C) under quiescent conditions.

Radiotracer studies were conducted with ^{14}C-chloroform and ^{14}C-dichloromethane to determine the ultimate fate of the substrate carbon, as well as to delineate pathways and stoichiometries. Interpretation of data required knowledge of Henry's constant for each substrate and product encountered. Therefore, peripheral studies were directed towards measurement of Henry's constants for 14 compounds over a temperature range from 10° to 35°C, and over a range of ionic strength from 0 to 1 M (KCl).

B. FINDINGS

PCE was completely converted to TCE in these studies, but the product was not further degraded to any significant degree. Other investigators (References 27 and 28) have indicated that PCE can be anaerobically degraded -- via TCE, dichloroethylene (DCE) isomers, and vinyl chloride (VC) -- to carbon dioxide. In this present research, only trace levels of 1,2-DCEs were detected. Experiments in which the starting compound was TCE showed only its slight conversion to DCEs and VC. Conditions were apparently unsuitable in this study —or a suitable organism (or consortium) was lacking in the inoculum — resulting in the apparent recalcitrance of TCE.

Results suggest that the microbially mediated reductive dechlorination of PCE is an inducible process. Approximately 40 days were required to effect conversion of PCE to TCE when the inoculum was first exposed to PCE — but nearly all of the conversion occurred over the final 3 days of that period. After incubation in the absence of PCE for 1 to 2 days, the seeded system required approximately 20 days to reacclimate to a subsequent dose of PCE, with PCE's eventual degradation occurring quite rapidly. The implication when a metabolic process is inducible is that the process is carried out for a purpose. In this case, the purpose remains unknown.

Microbial conversion of 1,1,1-trichloroethane (1,1,1-TCA) under anaerobic conditions did not require acclimation. A major portion of the compound (at least 40 percent) was reductively dechlorinated to 1,1-dichloroethane, which accumulated. The lack of an acclimation period suggests that 1,1,1-TCA is processed by a constituitive enzyme system (or at least one already functioning in this inoculum). In studies where 1,1,1-TCA was repeatedly restored to batch systems, the rate of its utilization remained unchanged following multiple additions. This implies that 1,1,1-TCA may not serve as a growth substrate for the mediating organisms. However, it is possible that their preexisting population might have been sufficiently high that the amount of growth occurring on the total, 9-micromole quantity of 1,1,1-TCA added over the seven repetitive injections was negligible by comparison, resulting in no apparent increase in utilization kinetics.

Preliminary investigation determined that 1,1,1-trichloroethane is a relatively reactive compound; for example, autoclaving (121°C, 30 minutes) a dilute solution in distilled water under aerobic conditions resulted in approximately 50 percent conversion of 1,1,1-TCA to 1,1-dichloroethylene via dehydrohalogenation. Therefore, the potential for reductive dechlorination of 1,1,1-trichloroethane under abiotic conditions was evaluated. Experiments with several chemical reducing agents — dithioerythritol, Fe(II), S(-II), and Sn(II) -- resulted in no detectable reduction of 1,1,1-TCA, suggesting that its abiotic, reductive dechlorination may not occur at ambient temperature and neutral pH.

Anaerobic, microbial utilization of chloroform (CF) proceeded without acclimation — again suggesting the involvement of a consituitive enzymatic system. Since CF is not a natural substrate, its metabolism may result merely from its resemblance to some other, commonly encountered substrate or metabolite. Results are unclear on the issue of whether the mediating microorganisms derive energy or other growth-related benefit from utilization of CF. The rate of utilization was proportional to the remaining CF concentration (to some positive exponent).

Approximately 31 percent of the degraded CF was converted to dichloromethane (DCM), which accumulated for an extended period before showing some evidence of slow decline. Far smaller quantities of chloromethane (CM) were also detected, especially during the subsequent, slow decline in DCM level. Methanethiol (MeSH) accumulated in relatively large amounts — amounts often significantly greater than that of the added CF. However, the direct source of MeSH is not CF or DCM. Results from this research, as well as the work of previous investigators, clearly demonstrate that MeSH accumulates (probably from methionine) because of the inhibition of methanogenesis by CF and/or DCM.

Studies with ^{14}C-chloroform indicated that 32 to 44 percent of added CF was converted to CO_2. The direct production of CH_4 from CF was not observed; however, it is possible that reducing equivalents released in the oxidation of CF to CO_2 were used to reduce CO_2 to CH_4. No evidence was obtained from this research to prove such a link between CF oxidation and methanogenesis.

Microbial degradation of DCM occurred readily after an acclimation period of variable length. Studies in which DCM was repeatedly added (as utilized) suggest that acclimation to and rapid utilization of this compound may be sustained indefinitely, so long as even a modest level of DCM is maintained in the system. However, the ability of a system to degrade DCM is rapidly lost in its absence; exposure to DCM-free conditions for as short a period as 4 hours resulted in a subsequent lag period of about 4 days. These results suggest that DCM degradation serves a purpose for the mediating microorganisms, and that an inducible enzyme system is involved.

Radiotracer studies demonstrated that the primary product of DCM degradation by the anaerobic culture was CO_2. Approximately 86 to 92 percent of the carbon from DCM was converted to CO_2. No direct conversion of DCM to CH_4 was detected. On the other hand, results show a definite link between DCM utilization and methane production. This suggests that the electrons removed in the oxidation of DCM to CO_2 are used to reduce some other compound (likely CO_2) to CH_4. A methanogenic system was definitely involved in the degradation of DCM; however, results from this research cannot yet prove the involvement of but a single class of organisms in the mineralization of DCM. For example, it is possible that DCM is oxidized by a H_2-evolving organism, with the H_2 used as electron donor in the reduction of CO_2 to CH_4 by a second group of microorganisms.

CF inhibits utilization of DCM in a manner that is slowly reversible. Inhibition persisted long after levels of CF in the solution were depleted. CF may actually cause death of DCM-utilizing microorganisms — or at least may bind almost irreversibly to important metabolic factors, persisting at low levels long after CF is otherwise removed. If this hypothesis is correct, then one of the only means of expediting the recovery of the DCM degradation process would be the introduction of fresh, uninhibited microorganisms after the freely available CF in the bulk solution has been removed. Such alleviation of inhibition was demonstrated in this research. Furthermore, inhibition of methanogenesis by CF may be the cause of DCM production from CF; reduction of CF to DCM may serve as a sink for reducing equivalents released from oxidation of some of the CF to CO_2. In the absence of

inhibition, these reducing equivalents might normally be channeled into the reduction of CO_2 to CH_4.

These findings have significance with respect to the potential use of a microbial system for renovation of a groundwater simultaneously contaminated by both CF and DCM. Biological treatment would appear to be most efficient if a sequential scheme were employed — with CF conversion accomplished to DCM and CO_2 in a first stage, followed by mineralization of DCM in the absence of CF in a second stage. Such a sequential process could, of course, be accomplished in a single, fixed-film reactor configuration; CF utilization could occur upstream of DCM utilization. Since the microorganisms would be attached to a solid support, the DCM users would not be exposed to CF.

Results from this research imply that in situ, biological treatment of groundwaters simultaneously contaminated by CF and DCM may be less effective than an aboveground facility — unless the requirements of the particular application allow the accumulation and continued presence of DCM in some subsurface locales. Conditions permitting, the entire subsurface system may be employed as a fixed-film reactor.

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PREFACE

This report was prepared for the Air Force Engineering and Services Center, Engineering and Services Laboratory, Tyndall AFB Florida, under Job Order Number (JON) 1900 7028. Funding for this effort was from the Air Force Office of Scientific Research. First Lieutenant Albert N. Rhodes was the AFESC/RD project officer.

The author conducted all research in the Engineering and Services Laboratory under the AFOSR University Resident Research Program. This is the final report for this project and covers technical efforts between June 1984 and June 1985.

This report has been reviewed by the Public Affairs Office (PA) and is releasable to the National Technical Information Service (NTIS). At NTIS, it will be available to the general public, including foreign nationals.

This technical report has been reviewed and is approved for publication.

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LIST OF ABBREVIATIONS

ASC	Autoclaved-Seed Control
CF	Chloroform
CM	Chloromethane
COD	Chemical Oxygen Demand
CT	Carbon Tetrachloride
CV	Coefficient of Variation
1,1-DCA	1,1-Dichloroethane
1,1-DCE	1,1-Dichloroethylene
cis-1,2-DCE	cis-1,2-Dichloroethylene
trans-1,2-DCE	trans-1,2-Dichloroethylene
DCM	Dichloromethane (Methylene Chloride)
E_H	Standard Reduction Potential
EPICS	Equilibrium Partitioning in Closed Systems
EtCl	Ethyl Chloride (Chloroethane)
FID	Flame-Ionization Detector
GC	Gas Chromatography
HS	Headspace
LS	Liquid Scintillation
LSC	Liquid Scintillation Counting
LSV	Liquid Scintillation Vial
MeSH	Methanethiol (Methyl Mercaptan)
MS	Mass Spectroscopy
ORP	Oxidation-Reduction Potential
PCE	Perchloroethylene (Tetrachloroethylene)
1,1,1-TCA	1,1,1-Trichloroethane
TCD	Thermal-Conductivity Detector
TCE	Trichloroethylene
TS	Total Solids
VC	Vinyl Chloride
VS	Volatile Solids
WC	Water Control

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SECTION I

INTRODUCTION

A. OBJECTIVE

The general goal of this study was to investigate the potential for degradation of five common, chlorinated, aliphatic hydrocarbons in anaerobic environments: tetrachloroethylene; trichloroethylene; 1,1,1-trichloroethane; chloroform; and dichloromethane. More specifically, attempts were made to delineate the importance of microbial -- as opposed to purely chemical -- mechanisms of degradation; products formed; pathways involved; and -- where possible -- the general categories of microorganisms involved.

B. BACKGROUND

Approximately 15-28 percent of all groundwater supplies in the United States contain synthetic organic chemicals (References 1-5). Among the most frequently detected contaminants are C_1 and C_2 chlorinated hydrocarbons such as tetrachloroethylene, trichloroethylene, 1,1,1-trichloroethane, chloroform, and methylene chloride (References 4 and 6). Most incidences of groundwater contamination by these and related compounds are suspected to have resulted from improper disposal of cleaning solvents, either through spreading on the land or at dump sites, although improper storage and accidental spills have been the indicated mechanisms in some cases. Ironically, homeowners on Long Island appear to have polluted their own groundwater through use of commercially available cesspool degreasing solvents containing trichloroethylene and methylene chloride (Reference 7). Although, in most instances, extremely low concentrations of contaminants are involved, there is concern that chronic exposure to chlorinated solvents could pose a carcinogenic or mutagenic health hazard (Reference 8).

The U.S. Air Force routinely uses trichloroethylene and other chlorinated solvents in the maintenance of aircraft; contamination of groundwater has been found in the vicinity of several Air Force bases.

The Air Force has since been actively participating in research addressing the technology of groundwater renovation, focusing on air stripping, carbon adsorption, and -- more recently -- in situ treatment involving microbial degradation processes.

Despite the ubiquity of the groundwater contamination problem and the great volume of research performed to date, information is lacking in one important subject area: the biotransformation/biodegradation of the common C_1 and C_2 chlorinated organic compounds. This lack of knowledge is particularly evident with respect to anaerobic microbial environments. Such information would be valuable for at least two purposes: models attempting to describe transport of C_1 and C_2 chlorinated compounds in the subsurface environment should consider biological transformation mechanisms; and it may be possible to alleviate the contamination problem via anaerobic microbial degradation of the pollutants, either in situ or through use of aboveground biological reactors, once the organisms involved have been studied and their requirements, capabilities, and limitations have been defined.

C. SCOPE

Five chlorinated compounds were studied separately in batch, anaerobic digestion experiments employing a diverse population of anaerobic microflora from a municipal waste treatment plant. The concentrations of the added, volatile substrates -- as well as the occurrence of volatile intermediates and products -- were routinely assayed using a headspace chromatographic technique. The added substrate concentrations ranged from 2-25 mg/L for the C_1 compounds, and from 1-2 mg/L for the C_2 compounds.

Radiotracer studies were conducted with ^{14}C-chloroform and ^{14}C-dichloromethane to determine the ultimate fate of the substrate carbon, as well as to delineate pathways and stoichiometries. Interpretation of data required knowledge of Henry's constant for each substrate and product encountered. Therefore, peripheral studies were directed towards measurement of Henry's constants for 14 compounds over a temperature range from 10° to $35^{\circ}C$, and over a range of ionic strength from 0 to 1 M (KCl).

SECTION II

BIODEGRADATION OF HALOGENATED ALIPHATICS

A. DEHALOGENATION

A large body of literature exists concerning the biodegradation of halogenated aromatic compounds because the persistence of halogenated pesticides and herbicides is of major concern (References 9-13). Considerably less information is available on biodegradation of halogenated aliphatics. Generally, however, halogens on aliphatic groups are more reactive than those on aromatic groups (Reference 14). Therefore, dehalogenation of aliphatics might be expected.

Dehalogenation can occur by any of several mechanisms: reductive dehalogenation, in which the halogen is directly exchanged for hydrogen;

$$CH_3\text{-}CCl_3 + H_2 \Rightarrow CH_3\text{-}CHCl_2 + HCl \quad (1)$$

dehydrohalogenation, in which the acid halide is removed without exchange;

$$CH_3\text{-}CCl_3 \Rightarrow CH_2{=}CCl_2 + HCl \quad (2)$$

replacement reaction with hydroxide, as mediated by halidohydrolases;

$$CH_2Cl_2 + H_2O \Rightarrow CH_2OHCl + HCl \quad (3)$$

or reaction with O_2, as mediated by oxygenases;

$$\rangle\text{-}Cl + O_2 + NADH + H^+ \Rightarrow \rangle\text{-}OH + HCl + NAD^+ \quad (4)$$

The actual mechanism in any instance will also be influenced by the compound being dehalogenated, as well as the microorganism(s) involved. Numerous biochemical factors have been indicated as influencing dehalogenation, including cytochromes, flavins, porphyrins, and inducible dehalogenating enzymes (Reference 15).

B. MICROBIAL INTERACTIONS IN DEHALOGENATION

Most of the chlorinated compounds of present concern with respect to groundwater contamination are not naturally occurring. Nonetheless, as aptly put by Ghosal et al. (Reference 16), "the structural plasticity of the genetic machinery of the microbes endows them with a degradative capability that can be quickly adapted in response to substrate variability." Rapid adaptation may result from minor alteration of existing enzymatic mechanisms (e.g., enzyme overproduction, inactivation of regulatory genes, or mutational divergence which results in production of enzymes with altered specificity). Alternatively, new enzymatic activities may result from recombination of genes for other enzymes or proteins (Reference 16). Transmissible-plasmid-associated genes have often been implicated in the degradation of halogenated compounds; it has been suggested that gene transfer among a mixed community of organisms capable of degrading a xenobiotic only through combined action might be expected to result in eventual consolidation of all the necessary genetic information within a single species (Reference 17).

Many instances of microbial degradation of xenobiotics are thought to result from cometabolism. The organism mediating the decomposition of the xenobiotic is not able to derive any direct benefit in terms of energy or cellular carbon; instead, it subsists on some other compound(s). Cometabolism is regarded as "accidental," reflecting a lack of substrate specificity by microbial transport and degradative systems (Reference 17).

In many -- if not most -- instances of microbial degradation of halogenated substances, extensive degradation of the compound requires a microbial consortium. This may be expected to be particularly true of anaerobic systems, where microbes are notoriously interdependent. For example, Suflita et al., working with enrichments from sewage sludge, determined that a three-membered methanogenic bacterial consortium was necessary (and sufficient) for the anaerobic degradadation of halobenzoates to CH_4 and CO_2 (Reference 18). Many similar instances are cited by Harder (Reference 17). In some cases, the consortium may be required because individual species are each capable of only partial decomposition of the substrate(s), thus, requiring the combined metabolic attack

by a series of microorganisms to effect complete mineralization. In other cases, the microbial interaction may be more subtle; mutualistic or commensalistic relationships based on growth-factor interdependence or removal of toxic products of metabolism are common. For example, Jensen (cited in Reference 17) isolated a three-species consortium, growing on trichloroacetic acid, which consisted of a primary, trichloroacetic-acid-degrading bacterium and two Streptomyces species. The latter two were unable to metabolize trichloroacetic acid, but apparently provided vitamin B_{12}, required by the primary organism. It is suspected that they subsisted on metabolites excreted from the primary organism.

Considering the complexity of microbial interaction often evident in systems found to degrade xenobiotic compounds, Harder has suggested that the use of a batch, pure-culture approach has serious drawbacks in the investigation of biodegradation potential (Reference 17). Continuous-culture enrichment methods are preferred, allowing the development of stable consortia whose interactions may then be properly investigated.

C. PREVIOUS INVESTIGATIONS OF MICROBIAL DEGRADATION OF C_1 AND C_2 CHLORINATED HYDROCARBONS

Considering the extent to which C_1 and C_2 chlorinated solvents are used, and the extent to which they have been released to the environment, it is suprising how few studies have been performed relating to their microbial degradation. Degradation under anaerobic conditions has been particularly neglected.

1. Aerobic Studies

Indirect evidence for the microbial degradation of C_1 and C_2 chlorinated hydrocarbons comes from several in situ studies. In situ degradation studies have been undertaken by many investigators with the aim of renovating contaminated groundwater systems through either the stimulation of indigenous microorganisms or the introduction of foreign microorganisms. In both instances, it is hoped that providing the

correct physical/chemical environment (i.e., correct pH via buffer addition, necessary nutrients, etc.) can stimulate microoganisms to decompose the contaminants of concern. In virtually all such studies, oxygen has been introduced to the subsurface environment (sometimes in the form of ozone or hydrogen peroxide). Thus, investigators have presumed that aerobic microorganisms are most effective for such tasks — despite the cost of oxygenation, and despite the body of laboratory evidence that, in many cases, anaerobic microbial systems appear able to degrade contaminants that are recalcitrant in aerobic systems. (But in fairness, it must be pointed out that there are probably compounds for which the reverse is true.)

The following study is typical of in situ investigations, and serves to illustrate the difficulty in attributing observed contaminant removal to microbial activity alone. In 1975, a leaking underground storage tank was discovered which had contaminated the subsurface with some 33,000 gallons of acetone, n-butyl alcohol, dimethylaniline, and methylene chloride (References 19 and 20). An aboveground, in situ treatment system was employed which consisted of a collection trench, a series of treatment tanks, reinjection trenches, and aeration wells. Contaminated groundwater was pumped to the tanks, nutrients (NH_4^+, ortho-P, Mg, Ca, Mn, and Fe) were added, and the resulting mixture was aerated for 16 to 18 hours before reinjection. After 1.5 years of operation, dramatic reduction in contaminant levels was evident in samples taken from observation wells. However, it is not known how much of the observed removal should be attributed to microbial decomposition, and how much was merely the result of air stripping. Many similar studies are cited by Lee and Ward (Reference 20). The simultaneous employment of nonbiological treatment methods and/or the lack of adequate experimental controls make it impossible to delineate the true role of microorganisms in the decontamination processes, at least in instances where removal of chlorinated C_1 and C_2 contaminants has been observed in situ.

Laboratory investigations of the potential for aerobic degradation of halogenated aliphatic hydrocarbons are few, but allow more definitive conclusions than do the in situ field studies. Bouwer et al. (Reference 21) incubated trihalomethanes, trichloroethylene, and

tetrachloroethylene (separately) at 9 - 85 ppb levels with an inoculum derived from municipal primary sewage effluent. Over 25 weeks of aerobic incubation, none of the compounds exhibited detectable utilization. However, several studies have indicated bacterial degradation of dichloromethane under aerobic conditions, implicating Pseudomonas spp. as the causative agents (References 22-25).

Brunner et al. (Reference 23) isolated a facultative methylotrophic bacterium (Pseudomonas sp. strain DM1) which could utilize dichloromethane, methanol, formate, mono-, di-, or trimethylamine as C_1 substrates. The investigators suggested that C_1 substrates are assimilated by the serine/isocitrate lyase-negative pathway. In a later publication from the same laboratory (Reference 25), Stucki et al. indicated that dichloromethane is directly attacked by a halidohydrolase, substituting OH^- for Cl^-, resulting in monochloromethanol which spontaneously forms formaldehyde. No activity was evident in cell extracts of DM1 or DM2 (a later-isolated strain).

LaPat-Polasko et al. (Reference 24) investigated secondary substrate utilization of dichloromethane by Pseudomonas sp. strain LP. Results demonstrated that the strain was capable of simultaneously consuming acetate and dichloromethane, when either was used as the primary substrate (ppm levels) in the presence of the other at ppb levels. This strain showed a preference for degrading dichloromethane over acetate.

Stucki et al. (Reference 25) derived two aerobic enrichments on 1,2-dichloroethane. A pure culture of bacteria (termed Pseudomonas sp. strain CE1), utilizing 2-chloroethanol, was isolated from one enrichment, prompting the speculation that a consortium is involved, with a pathway from 1,2-dichloroethane to glycolate via 2-chloroethanol, 2-chloroacetaldehyde, and monochloroacetate. The monochloroacetate might be expected to undergo dechlorination by a halidohydrolase to glycolate, which is readily utilizable by many different bacteria.

2. Anaerobic Studies

Among the few reported studies of chlorinated C_1 and C_2 compound degradation under anaerobic conditions are those of Bouwer et

al. (References 21 and 26). Both batch and continuous-column reactors were employed to investigate the possible degradation of several halogenated aliphatics, including trichloroethylene, chloroform, carbon tetrachloride, tetrachloroethylene, 1,1,1-trichloroethane, bromoform, dibromochloromethane, bromodichloromethane, and 1,2-dichloroethane, under anaerobic conditions at "environmental levels" (< 200 ppb).

Initial batch experiments were conducted with a methanogenic mixed culture as inoculum. Reactor bottles thus contained the halogenated compound(s) of interest, significant quantities of methanol used to prepare stock solutions of the halogenated substances, as well as residual substrates from the inoculum culture (Note: the inoculum was derived from a laboratory digester fed waste-activated sludge, xylan, and cellulose). Disappearances of chloroform, bromodichloromethane, dibromochloromethane, carbon tetrachloride, 1,1,1-trichloroethane, and tetrachloroethylene were noted from seeded reactor bottles, but not from sterile controls. This suggests biodegradation as the cause of disappearance. However, the sterile controls did not contain the biological and other solids contributed by the seed; hence, adsorption cannot be ruled out as a contributing mechanism in cases such as those of 1,1,1-trichloroethane and carbon tetrachloride, where disappearance was noted immediately. (However, later radiotracer studies with carbon tetrachloride convincingly demonstrated its conversion to CO_2). Nor can purely chemical degradation mechanisms be completely dismissed; there is no evidence that the oxidation/reduction potentials were comparable between seeded and sterile systems. Evidence of biodegradation is strongest in the cases of the brominated methane derivatives; subsequent spiking with the initially fed substrates resulted in rapid disappearance, suggesting acclimation. Likewise, disappearance of chloroform exhibited similar behavior, though only at the lowest (15 ppb) concentration. At 40 ppb, no convincing lag was evident, nor was acclimation very evident upon respiking.

In these early studies (References 21 and 26), evidence for biodegradation of trichloroethylene is not conclusive. Indeed, this compound was observed to accumulate in batch systems fed tetrachloroethylene, and appeared in the effluent of a fixed-film, acetate-enrichment culture receiving tetrachloroethylene.

Bouwer and McCarty (Reference 26) also performed experiments using radiolabeled compounds in batch and column reactors. ^{14}C-labeled chloroform exhibited approximately 86-98 percent conversion to $^{14}CO_2$.

No attempts were made by Bouwer et al. to isolate the causative organism(s), or to study their requirements and pathways. Many of their experiments had -- in addition to various halogenated organics -- acetate added as primary substrate, with methanol also present because of its use as solvent in preparation of stock solutions. Thus, these systems might best be described as acetate enrichment cultures. On the other hand, the systems were sufficiently mixed in population to prevent concluding that acetate-using methanogens are capable of degrading those compounds for which degradation was observed. Indeed, when methanogenic activity was inhibited with 2-bromoethanesulfonic acid, no loss of removal efficiency of halogenated compounds was observed, suggesting the possibility that methanogens play no role at all in the degradation of chlorinated organics. "Satellite" populations may exert a significant impact, given the low concentrations of halogenated compounds employed.

Subsequent studies have left little doubt that trichloroethylene can be degraded under anaerobic conditions. Vogel and McCarty (Reference 27) clearly demonstrated the transformation of tetrachloroethylene to trichloroethylene, dichloroethylenes, and vinyl chloride under anaerobic conditions in a continuous-flow methanogenic column fed acetate as primary substrate. In one instance, 24 percent of the influent tetrachloroethylene was mineralized to CO_2, with trichloroethylene as the major intermediate. In another instance, near quantitative production of vinyl chloride from tetrachloroethylene was observed.

Parsons et al. (Reference 28) observed the apparent conversion of tetrachloroethylene to trichloroethylene, cis-1,2-dichloroethylene, and vinyl chloride following extended incubation in microcosms containing muck from a recharge basin. No mass balance was possible, however. For example, from an initial mass of 100 micrograms tetrachloroethylene, 28 micrograms remained after 21 days; however, only 5 micrograms of trichloroethylene, 4 micrograms of cis-1,2-dichloroethylene, and 2 micrograms of vinyl chloride were accounted for. Some

of the "lost" material might reflect mineralization; on the other hand, production of undetected intermediates and sorption of any or all species, resulting in unpurgable fractions, may also have been significant.

D. SUMMARY

In summary, the anaerobic degradation of a variety of halogenated alipnatics has been demonstrated. In some cases — for example, those of chloroform and carbon tetrachloride -- the near quantitative conversion to CO_2 suggests biological oxidation, rather than reductive dechlorination. However, the appearancesof trichloroethylene, 1,2-dichloroethylenes, and vinyl chloride from degradation of tetrachloroethylene are clearly the result of reductive dechlorination. There is no conflict or contradiction in these observations; the mode of dechlorination doubtless varies among compounds (if not also among microbial systems). The pathways used by aerobic microorganisms to degrade chlorinated C_1 and C_2 compounds are better known than those of the anaerobic microbial systems. Since there are no steps in the known, aerobic pathways which require the action of molecular oxygen, anaerobic and aerobic pathways may be similar. This remains to be determined.

The organisms mediating the anaerobic degradation of chlorinated C_1 and C_2 compounds have not been identified; nor have pathways — and in many cases, intermediates — been described. In cases where intermediates have been observed (e.g., trichloroethylene from tetrachloroethylene), the stoichiometry has not been delineated. The possible contribution of nonbiological dechlorination mechanisms under reducing conditions has not been adequately addressed. And this author could find no reports in the literature concerning the anaerobic biodegradation of one very widely dispersed groundwater contaminant: methylene chloride (dichloromethane). This present study was undertaken in an attempt to shed some light on the above-described voids in our knowledge.

SECTION III

GENERAL PROCEDURES

A. PROGRAM OF STUDY

A series of related compounds was selected for study: tetrachloroethylene; trichloroethylene; 1,1,1-trichloroethane; chloroform (trichloromethane); and methylene chloride (dichloromethane). Some were expected to be intermediates in the degradation of others. For example, anaerobic degradation of tetrachloroethylene has been shown to yield trichloroethylene (References 26-28). Similarly, reductive dechlorination of chloroform might be expected to result in significant formation of dichloromethane.

These compounds were studied separately in batch, anaerobic digestion experiments employing a diverse population of anaerobic microflora as an inoculum. Though "starved" prior to use, the inoculum contributed unknown quantities of complex substrates to the batch systems. The concentrations of the added volatile substrates -- as well as the occurrence of volatile intermediates and products -- were routinely assayed with a headspace chromatographic technique. Two types of control systems were incubated with the viable, seeded systems: (1) autoclaved-seed controls (ASC), which were identical to viable systems except that the seeded reactor bottles were sterilized before addition of the chlorinated compounds; and (2) water controls (WC), which consisted of sterile distilled water plus the chlorinated compounds. All incubated systems consisted of 158.8 mL serum bottles with 100 mL liquid, sealed with Teflon™-lined rubber septa and aluminum crimp caps. All incubations were carried out at constant temperature (35°C) under quiescent conditions.

With 1,1,1-trichloroethane, where autoclaved systems evidenced some reductive dechlorination activity, experiments were subsequently undertaken to investigate the possible transformation of this substrate in sterile, defined systems over a range of oxidation potential. The

chlorinated substrate was added to sterile aqueous systems containing a variety of reducing substances which served as redox buffers.

Radiotracer experiments were conducted with ^{14}C-chloroform and ^{14}C-dichloromethane in an attempt to determine the ultimate fate of the substrate carbon, as well as to delineate pathways and stoichiometries of these two substrates. In the case of dichloromethane, enrichment and isolation of organism(s) responsible for its degradation were initiated.

To carry out the above-described experimental program, a number of preliminary experiments and procedural investigations were required. For example, it was necessary to demonstrate that headspace analysis is a viable, analytical tool for monitoring the course of degradation and the appearance of intermediates and products. Consequently, preliminary investigations were undertaken to ascertain the extent to which possible sorption or complexation of the C_1 and C_2 substrates might limit the use of GC headspace analysis as a means of estimating total quantities of substrates remaining and of products formed. Proper interpretation of headspace data requires that the resultant GC peak area from a 0.5 mL headspace injection be related to the total micromoles or nanomoles of a compound present in a reactor. Thus, calibration factors for each of 14 compounds (substrates and products) were determined experimentally. Likewise, interpretation of radiotracer data requires knowledge of Henry's constant for each compound encountered. Henry's constants were measured for 14 compounds, over a temperature range from 10° to 35°C, and over a range of ionic strength from 0 to 1 M (KCl). The EPICS procedure was employed, with slight modification (Reference 29).

This section contains the procedures and analytical methods which received wide application in this research. Descriptions of those procedures and methods that were peculiar to specific experimental studies are detailed in later sections, where appropriate.

B. ANAEROBIC SEED REACTOR

A diverse population of anaerobic microorganisms was desired for use as an inoculum in the batch degradation studies. Consequently, it

was originally supposed that freshly obtained, digested sludge from a local municipal wastewater treatment facility might be employed. However, headspace analysis of such material demonstrated the presence of numerous volatile substances yielding GC peaks which overlapped many of the compounds of interest to these studies. Therefore, this idea was abandoned. A compromise was struck between the use of "real" sludge (diverse, but already contaminated with volatile organics), and the use of a laboratory-generated inoculum which would be grown using synthetic substrates (clean, but perhaps not so diverse). Therefore, digested sludge was used to start the seed reactor, but a synthetic, complex substrate mixture was used to maintain it. Municipal digested sludge was added periodically to guarantee microbial diversity. This involved the removal of perhaps 20 percent of the reactor's contents, with addition of digested sludge to replace the lost volume. Such pericdic reseeding was done 1 to 3 weeks prior to use of the seed reactor's contents in a batch, anaerobic degradation study. The periodic addition of municipal digester sludge allowed the maintenance of species diversity (as evidenced by microscopic observations); and the intervening days or weeks of daily feeding, wasting, and gas production purged the reactor of interfering volatile substances.

The source of inoculum for the batch biodegradation studies was a 20-liter (15-liter liquid volume), stirred, anaerobic fermenter (VirTis™ Model 43-100) operated in semicontinuous mode with a 20-day retention time. The fermenter contained an internal coil system through which water from an external, thermostated bath was circulated, allowing controlled-temperature operation at 35 $\pm$ 1°C. A paddle system within the reactor was magnetically coupled to a variable-speed drive, allowing continuous mixing of the reactor under gas-tight conditions. Gas production from the seed reactor was measured with a wet-test meter.

The reactor was initially seeded with screened (0.25-inch mesh), municipal digester sludge obtained from the Millville Wastewater Treatment Plant, Bay County, FL. The feed to the seed reactor was based upon a commercial liquid food (Ensure™, Ross Laboratories, Columbus, OH) commonly used by hospitals for tube feeding. This commercially

available substrate has a labeled composition (COD basis) of 49 percent carbohydrate, 33 percent fat, and 18 percent protein. It is fortified with necessary vitamins and minerals to provide complete human nutrition (Table 1); however, from the standpoint of the nutritional needs of anaerobic bacteria, it is deficient in P, Mg, Co, and Fe. These, plus bicarbonate buffer, were added to a stock feed solution. Feed organic concentration was maintained at 10 g (COD)/L (Table 2).

Each day, the gas production reading from the wet-test meter was recorded; 750 mL of mixed liquor was removed from the reactor; and a feed volume of 750 mL was added. Thus, the nominal retention time of the reactor was 20 days. The only variations in this schedule occurred immediately after reseeding (when feed was withheld until gas production rates declined to normal reactor levels), or prior to use of the reactor contents as seed in a batch degradation study (before which feed to the seed reactor was also withheld to minimize the amount of exogenous substrate in the seed sludge).

The seed reactor's average performance and system characteristics are difficult to define in many respects, since the characteristics of the municipal digester sludge used to periodically reseed the unit varied over time. The seed unit's mixed liquor had a total solids (TS) content between 8.8 and 13.1 g/L, with only 48 - 51 percent volatile solids (VS) content (reflecting a poor degree of grit removal at the treatment plant because of extreme flow surges from newly-installed lift-station pumps). pH was very stable in the seed unit, never varying beyond the range from 6.9 to 7.05. Alkalinity averaged 3,050 (SD = 450) mg/L as $CaCO_3$. Typical gas production was about 4.5 L/day, except within the first few days after reseeding with municipal sludge, when values exceeding 9 L/day were often observed. Gas composition averaged 65 - 70 percent CH_4 and 35 - 30 percent CO_2.

C. REAGENTS

Fourteen volatile organic compounds were routinely encountered in this research. Some were added to batch reactor bottles in biodegradation experiments; others appeared as intermediates and/or products.

TABLE 1. LABELED COMPOSITION OF ENSURE™ LIQUID FOOD

Component	per liter		Component	per liter	
Protein	55.8	g COD	Choline	530	mg
Carbohydrate	155	g COD	Biotin	0.16	mg
Fat	107	g COD	Pantotnenic Acid	5.3	mg
Vitamin A	2640	IU	Sodium	850	mg
Vitamin D	210	IU	Potassium	1590	mg
Vitamin E	24.1	IU	Chloride	1440	mg
Vitamin K_1	0.038	mg	Calcium	530	mg
Vitamin C	159	mg	Phosphorus	530	mg
Folic Acid	0.21	mg	Magnesium	210	mg
Thiamine	1.6	mg	Iodine	0.079	mg
Riboflavin	1.8	mg	Manganese	2.1	mg
Vitamin B_6	2.1	mg	Copper	1.1	mg
Vitamin B_{12}	0.006	mg	Zinc	16	mg
Niacin	21	mg	Iron	9.5	mg

TABLE 2. CHARACTERISTICS OF INFLUENT TO SEED DIGESTER

Added Component[a]	per liter of feed	
Ensure™	31.5	mL
$NaHCO_3$	3360	mg
$K_2HPO_4 \cdot 3H_2O$	350	mg
$MgSO_4 \cdot 7H_2O$	200	mg
$FeCl_2 \cdot 4H_2O$	10	mg
$CoCl_2 \cdot 6H_2O$	7	mg

[a]Components added to Tyndall AFB tap water, with total hardness = 64 mg/L as $CaCO_3$, total alkalinity = 17 mg/L as $CaCO_3$, TDS = 160 mg/L, and pH = 7.1.

Calibration factors were required for each compound to convert observed GC peak area measurements into estimates of compound masses in reactor bottles; thus, each compound was required in relatively pure form. The compounds are listed in Table 3, along with synonyms, condensed chemical formulae, abbreviations used in this report, grades used, and commercial sources of each. Note that in many instances, the purity percentage was not specifically available, because the source was labeled as merely being "Reagent" or "ACS Grade."

D. BATCH ANAEROBIC DEGRADATION STUDIES

1. Experimental Strategy

Five parent compounds were selected for study: tetrachloroethylene (perchloroethylene, PCE); trichloroethylene (TCE); 1,1,1-trichloroethane (1,1,1-TCA); trichloromethane (chloroform, CF), and dichloromethane (DCM). The anaerobic degradation of each was investigated using a separate system of seeded sample bottles, autoclaved-seed controls, and distilled water controls for each compound. Usually, replicates of each type of bottle were employed. The batch studies were carried out using 158.8 mL serum bottles (Wheaton, borosilicate glass), sealed with 20 mm Teflon^{TM}-lined rubber septa (Supelco, Inc.) and aluminum crimp caps (Supelco, Inc.).

Seeded samples initially contained 100 mL of the digested sludge inoculum, plus one of the five chlorinated compounds. Autoclaved-seed controls were the same, except the bottles containing the sludge seed were autoclaved (121°C, 30 minutes) prior to addition of the chlorinated compound. The water controls initially contained 100 mL of distilled water, and were also autoclaved prior to the addition of the compound. The autoclaved-seed controls (ASC) served a number of purposes. By comparison with the water controls (WC), the extent of sorption by the sludge solids could be inferred (of course, there is no guarantee that sorptive characteristics of the sludge solids are unchanged by the autoclaving process); the comparison between ASC and WC systems also enabled detection of abiotic contributions to any observed transformations of the parent compounds. The contribution of

TABLE 3. VOLATILE ORGANIC COMPOUNDS AND THEIR SOURCES

Compound	Formula	Abbreviation	Grade and Source
Tetrachloroethylene (Perchloroethylene)	$Cl_2C=CCl_2$	PCE	Ultrapure 99+%, Alfa Products.
Trichloroethylene	$ClCH=CCl_2$	TCE	>99.5%, stablzd. w/ 0.01% triethylamine, Fluka AG Chem. Fab.
1,1-Dichloroethylene	$CH_2=CCl_2$	1,1-DCE	>99.9%, for GC, Riedel-De Haen AG.
cis-1,2-Dichloro-ethylene	cis-ClCH=CHCl	cis-1,2-DCE	97%, Aldrich Chem. Co.
trans-1,2-Dichloro-ethylene	trans-ClCH=CHCl	trans-1,2-DCE	>99.9%, for GC, Riedel-De Haen AG.
Vinyl Chloride (Chloroethylene)	$CH_2=CHCl$	VC	GC Std., 0.2 mg/mL in methanol, Supelco, Inc.
1,1,1-Trichloroethane	CH_3-CCl_3	1,1,1-TCA	Purified, inhibited, Fisher Scientific.
1,1-Dichloroethane	CH_3-CHCl_2	1,1-DCA	>98%(GC), Fluka AG Chem. Fab.
Chloroethane (Ethyl Chloride)	CH_3-CH_2Cl	EtCl	GC Std., 0.2 mg/mL in methanol, Supelco, Inc.
Tetrachloromethane (Carbon Tetrachloride)	CCl_4	CT	Analytical Reagent, Baker.
Trichloromethane (Chloroform)	$CHCl_3$	CF	ACS, preserved with 0.75% ethanol, Mallinckrodt.
Dichloromethane (Methylene Chloride)	CH_2Cl_2	DCM	Distilled in glass, for pesticide analysis, Burdick & Jackson.
Chloromethane (Methyl Chloride)	CH_3Cl	CM	GC Std., 0.2 mg/mL in methanol, Supelco, Inc.
Methanethiol (Methyl Mercaptan)	CH_3SH	MeSH	Reagent grade, Kodak.

active, anaerobic microbes to degradation of the chlorinated compounds was inferred by comparison between viable, seeded systems and the ASC systems.

To serve as suitable controls, the ASC systems should resemble the viable, seeded systems in every respect except one: viability. There is no feasible way to accomplish this; autoclaving unavoidably causes significant physical/chemical changes in the material, undoubtedly resulting in an altered environment. Addition of toxic chemical agents was initially investigated for possible use in preparation of nonviable controls. However, many candidates (e.g., chlorine, mercuric chloride) are oxidants whose use would result in unwanted changes in oxidation/reduction potential (ORP or E_H). Others, such as chloroform, would interfere with subsequent analysis of volatile, chlorinated compounds. Use of mercurous chloride was evaluated, with negative results (500 mg/L caused no significant reduction in gas production rates from samples of the anaerobic inoculum). Therefore, autoclaving was selected as the method for use in this research.

Comparison of pH and E_H values of the anaerobic inoculum before and after autoclaving showed little effect of the autoclaving process on these two environmental parameters (Table 4).

2. Procedures

The procedure for setting up a system of degradation bottles was as follows. First, distilled water was added to WC bottles, which were then sealed with septa and crimp-capped. Next, seed sludge was anaerobically transferred to a 2-liter culture flask, with constant purging by a N_2/CO_2 mixture at the same partial pressure of CO_2 as that of the seed digester (measured that very day using a GC with a thermal-conductivity detector [see Analytical Methods section below]). This purging was performed via a bent, stainless-steel cannula (14 cm by 1 mm ID) inserted into the culture flask, while simultaneously feeding the same N_2/CO_2 mixture into the headspace of the seed digester to replace the volume of seed being removed to the culture flask. To remove any traces of oxygen from the purge-gas mixture, it was passed through a bed

TABLE 4. E_H AND pH VALUES OF VIABLE AND AUTOCLAVED-SEED SLUDGES[a]

Description[b]	EH	pH
Unautoclaved sludge from seed digester (analyzed same day as removed from the digester)	-138 mV	6.95
Autoclaved sludge from seed digester (removed from digester, autoclaved, and analyzed that same day)	-141 mV	6.80
Unautoclaved sludge from seed digester (analyzed after 121 days of anaerobic incubation in serum bottle)	-141 mV	6.90
Autoclaved sludge from seed digester (removed from digester, autoclaved, incubated for 121 days, and then analyzed)	-131 mV	6.85

[a] E_H and pH values measured after sample bottles cooled to 25°C.

[b] In all cases, sludge samples were removed from the seed digester by anaerobic transfer to 158.8 mL serum bottles, which were then sealed under N_2/CO_2 purge prior to any cooling, autoclaving, or incubation steps.

of copper wire heated to 325°C before reaching the seed digester, the culture flask, or any batch degradation bottles.

The culture flask containing the anaerobic seed was then placed on a magnetic stirrer, while purging continued. This flask served as a reservoir from which serum bottles were filled. An automatic dispenser system was used to deliver the seed to serum bottles intended for eventual designation as either viable, seeded samples or ASCs. Tygon™ tubing led from the culture flask to the dispenser (a Unispense™ Model III, Wheaton Instruments) which was set to deliver 100 mL via a length of latex tubing to individual serum bottles. A parallel, seed/gas delivery system was employed which consisted of twin cannulae (each 10 cm by 1 mm ID, stainless steel) whose sides were soldered together at a point near the effluent ends of each. The purge-gas mixture was piped through one cannula, while the seed was delivered through the other. Thus, seed was anaerobically transferred to individual serum bottles by first inserting the parallel cannula system into a bottle; purging for approximately 30 seconds from one tube of the twin cannulae; pressing the start button on the dispenser, which initiated seed flow from the culture flask to the serum bottle via the other tube of the twin cannulae; and -- when the 100 mL volume of seed had been delivered -- carefully withdrawing the twin cannulae (continuing the purge) while simultaneously sliding a septum and crimp-cap seal into place. During transfer of seed, it was important to maintain a purge-gas flow rate to the culture flask that exceeded the liquid flow rate from the dispenser, to prevent introduction of air to the culture flask.

After introduction of inocula to viable, seeded-sample bottles and ASCs, the WC and ASC bottles were autoclaved, then cooled to ambient temperature. These, plus the viable, seeded systems, were then spiked with the parent compound of interest. This was accomplished using a 0.1 mL or 1.0 mL sterile syringe, depending upon the volume of stock solution to be injected. The stocks were saturated solutions of the individual chlorinated compounds in water, kept at ambient temperature. The volumes added ranged from 15 microliters to 500 microliters.

Actual amounts added to the bottles were 1 - 30 micromoles for the C_1 compounds, and 700 - 1400 nanomoles for the C_2 compounds.

Effort was taken to maintain sterile conditions in the ASC and WC systems. It would seem to make more sense if autoclaving were done after addition of the compounds, so that possible contamination could not occur upon insertion of the delivery needle. However, needles were to be inserted repeatedly, anyway, upon subsequent headspace sampling. Furthermore, preliminary studies indicated that autoclaving causes degradation of at least one compound: 1,1,1-trichloroethane. Thus, autoclaving had to be performed prior to addition of the chlorinated compounds.

Compound stock solutions were spread on nutrient agar plates; all proved negative, indicating that the likelihood of significant contamination originating from the stock solutions was not great. Contamination from insertion of the needles was a greater concern. Precautions were taken to sterilize the needles (with a furnace coil) both before and after additions to each ASC and WC bottle. Similar precautions were taken with subsequent headspace samplings; the syringe needles were sterilized before penetration (but were cooled completely to avoid contamination of the needle by products released in searing the rubber septa). The syringes were never pumped or otherwise discharged before use. Nonetheless, there is no guarantee that ASC and WC systems remained sterile. The experimental objective required only that they remained sufficiently free of contamination long enough for insignificant transformations of the parent compounds to be observed in ASC and WC systems over a time period in which significant transformations were observed in the viable, seeded systems. Under these conditions, biologically mediated mechanisms could be inferred as being the cause of transformation in the viable systems.

After spiking all appropriate bottles with the parent, chlorinated compounds, the serum bottles were quiescently incubated at 35°C. Periodically, bottles were removed for headspace analysis. At such times, they were placed in a reciprocating shaker bath (Precision Shaking Water Bath) at 35°C. The bottles were held in place within the

bath by spring-steel clips attached to the shaker platform, and the water level in the bath was maintained so that the tops of the serum bottles protruded approximately 1 cm above the surface, ensuring that both the headspaces and liquid contents of bottles would be maintained at 35°C for proper analysis of headspace concentrations. While in the bath, the bottles were gently shaken (approximately 60 cycles per minute, at a throw of 1.2 cm) -- mostly to agitate the bath water, minimizing temperature gradients. Except for this period of mixing — and the vigorous shaking which followed any respiking of a bottle with additional stock solution — the bottles were quiescently incubated.

E. HEADSPACE ANALYSIS OF VOLATILE COMPOUNDS

1. Preliminary Considerations

The degradation of five compounds was studied, with each expected to yield numerous volatile intermediates and volatile products. Considering that detection and quantification of each and every such compound was desired, the analytical problem was a difficult one.

Preliminary studies were undertaken to invesigate analytical options. Direct headspace injection, as well as preliminary liquid/liquid extraction (with subsequent liquid injection of the solvent extracts), were both evaluated. Studies with three different gas chromatographic (GC) columns and several possible extraction solvents (pentane, toluene, and carbon disulfide) indicated that headspace chromatography should be employed if at all possible. Headspace sampling and injection is much less consuming of sample; a single bottle can be repeatedly analyzed without removing any of the bottle's liquid contents. Only a relatively small portion (0.5 mL) of the bottle's headspace (58.8 mL) is removed each time.

On the other hand, the use of a headspace analytical technique prompted a number of concerns — some peculiar to use of the headspace procedure, and some of equal concern regardless of the analytical method employed: (1) Sorption of the added compounds by the seed solids might

be appreciable, making it apear that degradation had occurred when, in fact, it had not (Note: the headspace concentration is proportional to the free -- unsorbed, uncomplexed -- mass of volatile compound in the bottle); (2) repeated puncturing of incubation bottle septa may cause substantial loss of volatile compounds, either by passage through the septa or by sorption onto the rubber beneath the punctured, Teflon™ lining; and (3) the large concentration of methane expected in bottle headspaces may severely mask the presence of other low-molecular-weight, volatile intermediates and products, since the retention of methane by a GC column is not expected to be much different from that of such likely intermediates as chloromethane or vinyl chloride. Because methane may comprise up to 70 percent of the bottle headspace, this could be a severe limitation.

The possible sorption of the chlorinated compounds by the seed solids was investigated by individually adding each of the five study compounds in various concentrations (from 0.02 to 2.0 mg/L) to serum bottles containing water only, and to bottles containing the seed digester mixed liquor at approximately 9 g TS/L. Bottles were allowed to incubate for only 1 to 2 hours, to minimize any possible effects of microbial uptake.(Note: Alternatively, the experiments could have been performed using autoclaved, digested sludge solids, but the sorptive properties of autoclaved solids may differ from those of viable solids). Headspace concentrations* were compared between water and sludge systems to which identical compound additions had been made. Results indicate no significant sorption of the five chlorinated compounds, with the exception of tetrachloroethylene, for which approximately 20 percent of the compound added was lost from free solution and apparently sorbed. However, even in this case, a near perfect linearity was found between headspace GC peak area and added compound concentration (Figure 1). Thus, it appears that sorption does not preclude the use of the headspace technique for routine monitoring of batch degradation systems. In any event, ASC systems were available in virtually all anaerobic

* 0.5 mL headspace samples taken at 23°C from bottles incubated at this temperature. GC column was 4.3 meters by 3.2 mm, 10 percent SP-1000 on 100/120 Supelcoport™, isothermally operated at 100°C.

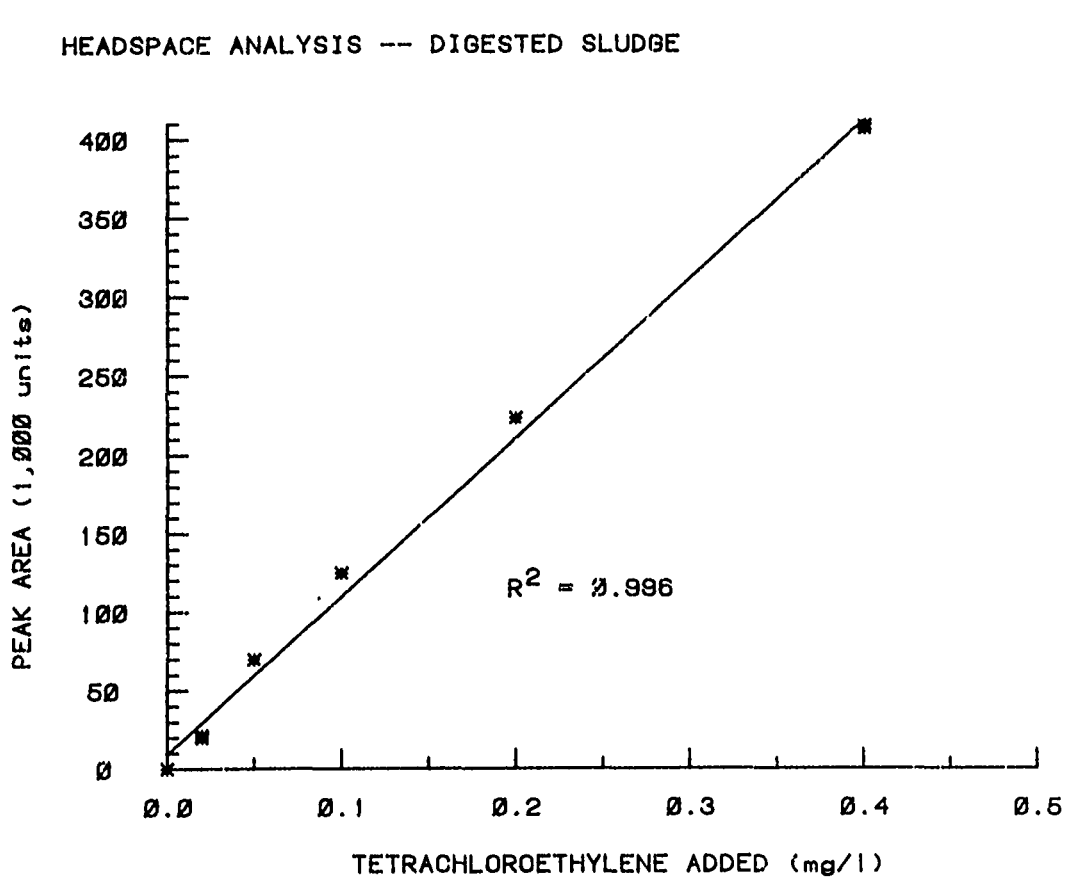

Figure 1. Headspace Analysis of Tetrachloroethylene After Standard Additions to Digested Sludge.

degradation studies, partly to assess the extent of sorption by comparison with WC systems.

The significance of losses from incubated bottles was investigated by the addition of each parent compound to separate serum bottles containing 100 mL (autoclaved) distilled water. (Note: A sixth compound, carbon tetrachloride, was also investigated because it was originally intended as a substrate for the degradation studies along with the other five). In previous research, losses of volatile compounds through or within septa had been appreciable (e.g., see References 30 or 31). One might expect either sorption or diffusive loss to be more significant from the gas phase than from the aqueous phase; thus, these bottles were incubated while on their sides — with the aqueous phase contacting the septa. Bottles were incubated at room temperature (ca. 23°C) for 40 days, with periodic headspace samples routinely taken for GC analysis. Results are depicted in Figure 2, and indicate that losses tend to be less than or equal to 10 percent over this incubation period — certainly an acceptable degree of loss. In any event, WC systems were available from virtually all the anaerobic degradation studies to assess the significance of losses.

2. Analytical Method

The headspace monitoring technique appeared workable. An analytical method was next sought to detect and quantify the numerous volatile compounds expected to be present in incubated sample systems. After much experimental investigation, the following method was selected.

A 2.44-meter by 3.2 mm stainless-steel GC column was used, packed with 1 percent SP-1000 on 60/80 Carbopack-B™(Supelco, Inc.). Injector temperature was 200°C; detector temperature, 250°C; carrier flow (He) was 40 mL/min; and a flame-ionization detector (FID) was employed with hydrogen and air flows of 40 and 400 mL/min, respectively. The following temperature-programming sequence was used: 60°C for 2 minutes; 20°C per minute to 150°C (with no hold); and 10°C per minute to 200°C, with a 4.2-minute hold at this temperature. This program consumed 15.7 minutes; considering the recycle (oven-cooling) time,

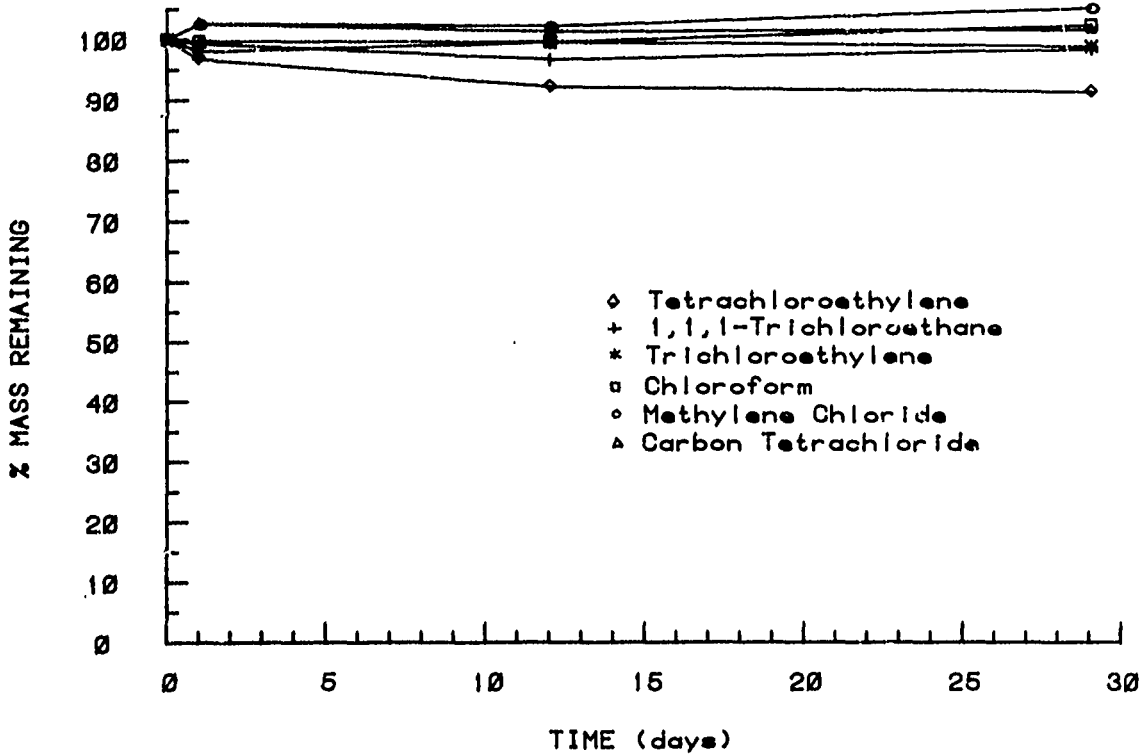

Figure 2. Loss Studies (Compounds in Distilled Water).

approximately 23 minutes were required between consecutive sample injections. All GC analyses were performed with a Sigma 2000 Gas Chromatograph (Perkin-Elmer), coupled with an LCI-100 Laboratory Computing Integrator (Perkin-Elmer). With this GC method, excellent resolution was obtained between all compounds detected, a reasonable analytical time was achieved, and yet the methane peak — which eluted first — did not interfere with any (known) peak of interest. In Table 5, the retention times of all the assayed compounds are shown. Metabolites are grouped together with their corresponding parent (added) compounds.

The sampling procedure was as follows. When analyses were to be performed, serum bottles were transferred from the incubator in which they were stored (at 35°C) to the reciprocating shaker bath at 35°C, where they were held by flask clamps in water to within 1 cm of their tops. Sampling was thus accomplished while headspaces remained at 35°C. A 1.0 mL gas-tight syringe (Pressure-Lok™, Series A-2) with a push-button valve mechanism was used to obtain 0.5 mL headspace samples. This syringe was fitted with a 5 cm (0.71 mm OD by 0.46 mm ID) side-port needle. The syringe needle was inserted through the bottle septum; a 0.5 mL sample was drawn in; the push-button valve was closed; the syringe needle was withdrawn from the bottle and inserted through the GC septum; and in closely synchronized steps, the push-button valve was opened and the sample was immediately injected.

Use of a syringe with a push-button valve became necessary because of the likelihood that headspace pressures in serum bottles would often exceed that of the atmosphere. In such cases, removal of an unlocked syringe needle from the serum bottle would cause a portion of the gas within the syringe sample to be expelled, resulting in loss of a portion of the volatile compound. Since what was desired was a measure of the gaseous concentration of the compound as it existed in the serum bottle, it was desirable to inject it at serum-bottle concentration — and that meant at serum-bottle pressure.

TABLE 5. COMPOUNDS DETECTED AND THEIR GC RETENTION TIMES

Parent Compound	Additional Compounds	RT (minutes)
Tetrachloroethylene (PCE)		14.5
	Trichloroethylene (TCE)	10.1
1,1,1-Trichloroethane (1,1,1-TCA)		8.51
	1,1-Dichloroethane (1,1-DCA)	6.86
	Chloroethane (EtCl)	4.05
Trichloroethylene (TCE)		10.1
	1,1-Dichloroethylene (1,1-DCE)	6.30
	cis-1,2-Dichloroethylene (cis-1,2-DCE)	7.08
	trans-1,2-Dichloroethylene (trans-1,2-DCE)	7.13
	Vinyl Chloride (VC)	3.39
Chloroform (CF)		7.35
	Methanethiol (MeSH)	2.75
	Dichloromethane (DCM)	5.08
	Chloromethane (CM)	1.60
Dichloromethane (DCM)		5.08
	Methanethiol (MeSH)	2.75
	Chloromethane (CM)	1.60

3. Identification of Unknowns

Preliminary incubations with seeded cultures defined the compounds to be expected from the anaerobic biological degradation of the five chlorinated compounds selected for study (Table 5). As new, unknown peaks were detected from headspace analysis, they had to be identified. The objective was the eventual calibration of GC peak areas verus moles of (free) volatile substances present in the bottles.

Identification of unknown compounds was accomplished by gas chromatography/mass spectroscopy (GC/MS), using a GC column and GC method identical to that employed in the routine headspace analyses. The GC/MS system was composed of a Finnigan Model 3500 GC/MS, coupled with a Teknivent Interactive GC/MS Data System. Identification was always confirmed by injection of standards of the supposed compound into the same GC system routinely used for headspace monitoring. In some cases, due to differences in detection limits between the MS and FID detectors, the MS system could not be used to identify the unknowns. (Note: With one compound of interest, methanethiol, a heated transfer line from the GC to the MS apparently caused the thermal conversion of MeSH to dimethylsulfide, resulting in the initial misidentification of MeSH). In such cases, identification was accomplished by matching retention times of unknowns with standards. Two different GC columns were used: the analytical column described earlier, and a 4.3-meter by 3.2 mm ID stainless-steel column packed with 10 percent SP-1000 on 100/120 Supelcoport^{TM} (Supelco, Inc.). Matching retention times with both columns was considered confirmation of the unknown's identity.

4. Calibration

Thirteen compounds were identified in batch cultures (Table 5). Calibrations of GC peak areas versus moles present in serum bottles were then effected for all 13. The method involved the addition of known masses of each to bottles identical to those used in the incubation studies, but containing 100 mL of distilled water, instead of the anaerobic seed. Triplicate bottles were used for each compound; an

equilibration period of 18 hours was employed, with bottles held at 35°C both during the incubation, as well as during sampling of headspaces for injection. The coefficients of variation for the calibration factors (relating peak areas to nanomoles or micromoles of compound present) were typically 1 percent. This calibration procedure was performed in conjunction with determinations of Henry's constant for these compounds (and a 14th compound, carbon tetrachloride). Procedures and results are detailed in Appendix A.

F. ANALYSIS OF ^{14}C-LABELED ORGANIC COMPOUNDS

1. Experimental Strategy

For two substrates, chloroform (CF) and dichloromethane (DCM), degradative pathways were examined through use of radioisotopes. An aqueous stock solution of chloroform containing 1.264×10^6 dpm/mL ($\pm$ 0.65 percent coefficient of variation [CV]) was available from R. G. LaPoe (Reference 30). This radiolabeled CF had been obtained originally in "neat" form (New England Nuclear, purity >99 percent; specific activity = 4.6 mCi/mmol) in a break-seal ampoule, from which the aqueous stock was prepared using oxygen-free distilled water. The ^{14}C-CF stock was contained in a 158.8 mL serum bottle, sealed with a Teflon™-lined rubber septum and aluminum crimp cap, and stored in a refrigerator when not in use. In the case of DCM, the radioisotope was not readily available. Fortunately, CF was partially converted to DCM in the anaerobic, batch degradation systems; hence, the final products of DCM degradation could be inferred from the fate of the ^{14}C-DCM which was microbially produced from ^{14}C-CF.

After addition of radiolabeled CF to seeded, anaerobic degradation systems, the fate of the ^{14}C material was evaluated using a sequence of analytical steps designed to quantify the amount of ^{14}C in each of several fractions: CF, DCM, CM, MeSH, CH_4, CO_2, and a category of residue operationally defined as "nonstrippable at pH 4.4." This residue was further fractionated to yield estimates of soluble- and solid-phase ^{14}C associated with it.

2. Analysis of ^{14}C-Labeled Volatile Organics

a. Method

The ^{14}C associated with CF, DCM, CM, or MeSH was quantified by trapping fractions in the effluent from a GC column into liquid-scintillation (LS) cocktail. The column and GC operating conditions were identical to those described in Section III-E-2. The only difference was that the effluent end of the column was disconnected from the FID, and attached to a 0.3-meter section of thick-walled, 3.2 mm (OD) stainless-steel tubing, which served as a conduit from the GC column oven to the outside, while allowing almost complete closure of the oven door. This conduit was attached via an Ultra-Torr™ fitting at its effluent end to a 4.8 mm (OD) glass tube, terminating in a porous-glass diffuser of ASTM porosity C (25-50 micrometers). Since the GC conditions — including carrier flow rate — were identical to those employed for routine headspace analyses, the retention times of CF, DCM, CM, and MeSH were well-known and sufficiently different to allow the separate trapping of each (see Table 5).

A 0.5 mL headspace sample from one of the anaerobic degradation systems was collected (at 35°C) and delivered to the GC as per Section III-E-2. At the appropriate time, a 20 mL glass liquid-scintillation vial (LSV) containing 15 mL of cocktail (Beckman MP) was held in place so that the diffuser from the trapping system was fully immersed. For a 1-minute period, the column effluent was allowed to pass through the cocktail, after which the LSV was sealed with a plastic screw cap. The collection periods used for each compound were as follows (Note: times refer to minutes after injection): CM, 1.50-2.50; MeSH, 2.50-3.50; DCM, 4.75-5.75; and CF, 7.20-8.20. With every analysis, control (baseline) data were obtained by running through the GC program without making an injection, and then trapping the GC column effluent over the same intervals as those used for the corresponding sample assays. The data so-obtained thus represent baseline values which could be used to correct sample data for slowly-eluting ^{14}C contaminants in the GC column which may have accumulated over the many weeks of injecting headspaces from degradation systems receiving radiolabeled

substrate. In no instances were baseline dpm values significantly above dpm levels of cocktail blanks.

The cocktail traps were then analyzed for ^{14}C using a liquid scintillation counter (LSC) as described in a later section. The dpm data obtained in this manner represented the radioactivity levels of the individual, trapped compounds in 0.5 mL headspace samples at 35°C. The total dpm contributed by a compound in the serum bottle from which the headspace sample was derived was then calculated:

$$dpm_{tot} = 2(\text{dpm per 0.5 mL HS sample})[58.8 + (100/H_c)] \qquad (5)$$

where H_c is the applicable dimensionless Henry's constant for the compound at 35°C; 58.8 = mL of headspace (HS) in the reactor bottles; and 100 = mL of liquid volume. Values of H_c used were those experimentally determined in this research (see Appendix A for details).

b. Evaluation of Method

^{14}C-chloroform was the only radiolabeled compound available in purified form for use in the direct evaluation of the efficiency of the GC effluent-trapping method. Direct measurement of trapping efficiency was accomplished by addition of 98 mL of distilled water to a 158.8 mL serum bottle, which was then sealed with a septum and aluminum crimp cap. Using a syringe, 2 mL of the ^{14}C-CF stock was injected into the serum bottle, which was then equilibrated at 35°C for several hours in a shaker bath. A 15 mL volume of MP (Beckman Instruments) LS cocktail was dispensed to each of two LSVs. One of the vials was fitted with a Teflon™/rubber septum, held in place by a conventional, plastic LSV cap through which a 3 mm hole had been drilled. The other LSV was to be used in conjunction with the GC-effluent trapping method. A 0.5 mL headspace sample from the serum bottle containing the ^{14}C-CF was injected directly into the headspace of the septum-sealed LSV, which was then shaken to dissolve the radioisotope. Another 0.5 mL headspace sample from the serum bottle was injected into the GC, for analysis of the ^{14}C-CF via the trapping method. Both vials were analyzed by LSC, and results were compared (after correction for background and quench level).

This direct method of evaluating GC-effluent trapping efficiency was employed on three different occasions, with efficiencies of 88.0, 98.0, and 94.7 percent observed.

For DCM, CM, and MeSH, direct measure of trapping efficiency was not possible. Indirect evidence is available from analyses of headspaces in bottles whose pH values had been elevated to >10.5 (by NaOH addition) for $^{14}CH_4$ analysis. At this high pH, virtually all $^{14}CO_2$ was eliminated from headspaces; given that subsequent analyses demonstrated the presence of negligible $^{14}CH_4$ quantities, then virtually all dpm in the bottle headspaces at high pH were probably attributable to ^{14}C-DCM, and/or ^{14}C-CM, and/or ^{14}C-MeSH. Hence, dpm values from direct injection of headspace samples (from serum bottles at high pH) into serum-capped LSVs could be compared with the sums of dpm values observed from GC-effluent trapping. This indirect estimation of trapping efficiency was made on two separate occasions, employing two different anaerobic degradation bottles which contained measureable ^{14}C-DCM, ^{14}C-CM, and ^{14}C-MeSH, but negligible $^{14}CH_4$ or ^{14}C-CF. GC-effluent trapping efficiencies of 92.7 and 82.6 percent were observed.

A further concern with the GC-effluent trapping method was the possibility that $^{14}CO_2$ might elute from the GC column with a retention time overlapping that of a compound of interest. Although unlikely, this possibility was investigated. Because the FID detector does not respond to CO_2, the analytical column was connected to a thermal-conductivity detector (TCD). Using the GC system parameters and temperature program described above, a 0.25 mL injection of CO_2 indicated that the retention time for this compound was 0.63 minutes -- far below that of any other ^{14}C-labeled compound encountered in these studies.

3. Analysis of $^{14}CH_4$

a. Method

The LS cocktail does not have sufficient affinity for methane to allow its assay by the GC-effluent trapping method employed with the other volatile organics. Consequently, a different method was

used for quantification of $^{14}CH_4$ produced in anaerobic degradation systems. The method was based upon the combustion of $^{14}CH_4$ to $^{14}CO_2$ (in the absence of other, interfering, ^{14}C-labeled organics), with subsequent trapping of the $^{14}CO_2$ into an alkaline LS cocktail.

The first step in the analysis of radiolabeled methane was the injection of 1.5 mL of 5 N NaOH to the anaerobic degradation system, raising the pH above 10.5 to absorb virtually all $^{14}CO_2$ from the headspace. The 1.0 mL gas-tight syringe and sampling procedure that were earlier described (Section III-E-2) were next used to obtain a 1.0 mL headspace sample at 35°C for $^{14}CH_4$ analysis. Immediately upon sampling (i.e., prior to withdrawal of the needle from the reactor), the push-button valve on the syringe was closed. The needle was then removed from the syringe, and a Tenax™ trapping system was installed in its place (Figure 3). This trap consisted of a plastic, check-valve tee (Pall) (fitted with a Teflon™ sleeve to provide a gas-tight union with the syringe), followed in series by a 4 cm length of 6.4 mm (OD) plastic tubing, a 3 cm disposable, plastic pipet tip, and a 5 cm side-port needle. The trap contained approximately 0.1 g Tenax™, packed between two glass-wool plugs. The purpose of the Tenax™ trap was to remove radiolabeled organic compounds (other than methane) during injection of the headspace sample to the $^{14}CH_4$ combustion chamber (Figure 4).

The combustion chamber was constructed from inner and outer \$29/42 Pyrex™ joints (3 cm OD), fitted with a septum-sealed side arm and two 6.35 mm (OD) needle-valve tee connections (Hi-Vac™ Teflon™, Kontes Glass Co.). The inner joint was sealed around two platimum wires, to which a nichrome heating coil (7 ohms at ambient temperature) was silver-soldered. The other ends of the platinum wires were soldered to two insulated leads which were attached to a variable voltage source (0 - 120 Vac) via alligator clips. The gas volume of the combustion chamber (measured with the needle valves closed) was 47.2 mL. A source of high-purity oxygen was connected to the lower tee; the upper tee led to a porous-glass diffuser (ASTM porosity C, 25-50 micrometers).

Prior to injection of a headspace sample, the combustion chamber was purged for several minutes with oxygen, then sealed by closing the two needle valves. The 1.0 mL headspace sample was then

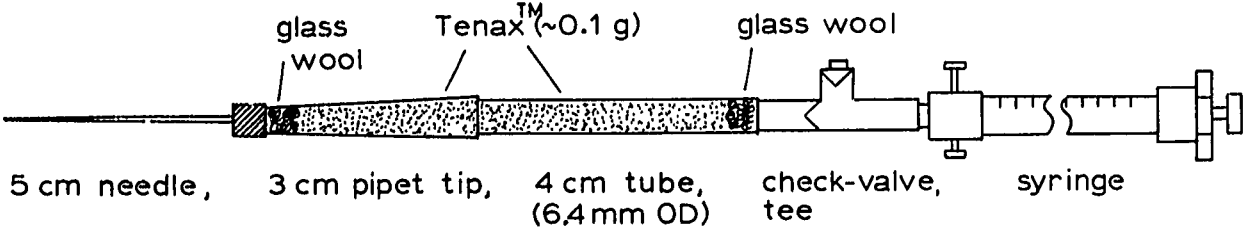

Figure 3. Tenax™ Trap System For Injection of Headspace Samples to Combustion Chamber.

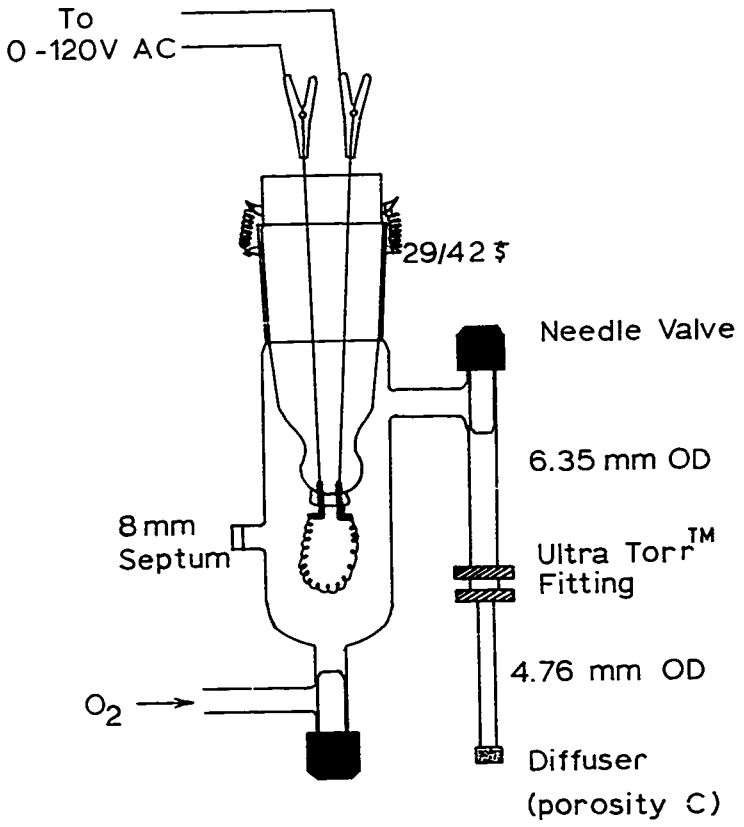

Figure 4. Combustion Chamber.

delivered via the Tenax™ trap as follows: the needle was inserted through the septum of the combustion chamber; the syringe valve was opened; the sample was delivered slowly (perhaps over 10 seconds) while one finger was held over the unattached member of the syringe trap's check-valve tee (as insurance against possible leakage of sample from the check valve under such low flow conditions); and, finally, delivery of the full 1.0 mL headspace sample was ensured by subsequently flushing an additional 4.0 mL of room air slowly through the trap system, into the combustion chamber. This flushing was accomplished by repeatedly drawing room air into the syringe via its unattached check valve, followed by slow delivery to the chamber.

Combustion of $^{14}CH_4$ to $^{14}CO_2$ was accomplished by applying 15 volts (ac) to the nichrome coil for 60 seconds. This voltage caused the coil to glow brightly. A 20 mL glass LSV containing 15 mL of MP cocktail plus 0.1 mL of monoethanolamine was positioned such that the diffuser from the combustion chamber reached to the bottom of the alkaline cocktail. The needle valves were opened, and the chamber was purged with an oxygen flow of 50 mL/min for 10 minutes, with the effluent continuously passing through the alkaline cocktail trap. Afterwards, the LSV was sealed with a screw cap and analyzed by LSC. Data were corrected for background dpm by making a 1.0 mL injection of room air to the combustion chamber (via the syringe-trapping system) and performing the combustion/collection procedure exactly as for samples. This background analysis was performed prior to any sample analysis; in all cases, background radiation levels (typically 35-45 dpm) were not significantly above values recorded for alkaline cocktail blanks (33-35 dpm). The total dpm attributable to $^{14}CH_4$ in an anaerobic degradation system was calculated by multiplying the dpm value measured in an alkaline cocktail trap (after subtraction of background dpm) by 57.3 (i.e., the headspace volume, in milliliters).

b. Evaluation of Method

Since no purified $^{14}CH_4$ was available, evaluation of this method had to rely on a set of experiments, each designed to evaluate only a portion of the analytical sequence.

The objective behind the addition of alkali to reactor bottles prior to headspace sampling was to absorb $^{14}CO_2$ from the headspaces, eliminating one source of potential, positive error in the subsequent analysis of $^{14}CH_4$. To determine the efficiency of CO_2 absorption, a 1.0 mL headspace sample was taken from every bottle being analyzed for $^{14}CH_4$, after the NaOH had been injected. These samples were analyzed for CO_2 via GC, using a TCD. No detectable levels of CO_2 were observed. The detection limit for CO_2 was approximately 5×10^{-5} atm -- roughly the same partial pressure as would be predicted in the serum bottles, given their pH (10.5) and C_T (0.072 M). Under these conditions, the CO_2 in the headspace after NaOH addition should represent less than 0.002 percent of the total carbonate species present.

The Tenax™ syringe trap was employed to sorb radiolabeled organics other than $^{14}CH_4$, which must be quantitatively passed through the system for the method to be accurate. The lack of affinity of the syringe trap for methane was evaluated as follows. A 60 mL serum bottle (containing only room air and a small, Teflon™ gas-mixing tumbler) was sealed with a 20 mm Teflon™-rubber septum and aluminum crimp cap. A 1.0 mL quantity of purified methane was injected into this bottle, using a conventional syringe with push-button valve. The bottle was shaken to mix the gases, and a 1.0 mL sample was analyzed by GC (TCD). The bottle was then opened, flushed with an air stream, and the procedure was repeated, with one difference: this time, the purified methane was injected to the serum bottle via the Tenax™ syringe trap (with postinjection flushing). Comparison of GC peak areas indicated that > 98 percent of the methane was delivered through the syringe trap.

The ability of the syringe trap to sorb CF and DCM was evaluated as follows. A CF/DCM stock mixture was prepared in a 158.8 mL serum bottle by adding 3 mL of CF-saturated water and 3 mL of DCM-saturated water to 100 mL of distilled water, then sealing as usual. This bottle was then shaken for 30 minutes while equilibrating at ambient temperature. The same procedure as described in the preceding paragraph (for evaluating the retention of methane by the syringe trap) was employed here for CF and DCM. One-milliliter headspace samples from the CF/DCM stock bottle were injected into the 60 mL serum bottle --

with and without use of the syringe trap. Subsequent 0.5 mL samples from the serum bottle were analyzed via GC (FID); results indicated no detectable CF or DCM when the syringe trap had been used. Considering the detection limits of the GC technique, it can be stated that retentions by the syringe trap exceeded 99.8 percent and 99.5 percent for DCM and CF, respectively.

The ability of the syringe trap to retain MeSH was not investigated. In the first place, MeSH behaves as a weak acid. Lange's Handbook (Reference 32) reports pK_a = 10.7 at 25°C. The value at 35°C, corrected for ionic strength, would be lower. Thus, in anaerobic systems to which NaOH has been added (pH > 10.5), considerable reduction of MeSH headspace concentration via aqueous absorption is expected. Secondly, GC-effluent trapping demonstrated only insignificant ^{14}C-MeSH in the anaerobic systems, making its removal prior to $^{14}CH_4$ analysis unnecessary. Similarly — for this latter reason — the retention of CM by the syringe trap was not evaluated.

The efficiency of the combustion step was evaluated as follows: The chamber was flushed with oxygen; needle valves were closed; 0.5 mL of purified methane was injected through the chamber septum; after waiting a few minutes, a 1.0 mL gas sample was analyzed via GC (TCD); power was applied to the combustion coil for 60 seconds; and a second 1.0 mL gas sample was analyzed. Comparison before and after combustion indicated complete conversion of CH_4 to CO_2, at least within the detection limits of the GC analysis.

The efficiency of the procedure used to purge the chamber after combustion and to trap the radiolabeled material in alkaline cocktail was evaluated. Since neither $^{14}CH_4$ nor $^{14}CO_2$ were available in purified form, ^{14}C-labeled CF was employed. A 0.5 mL headspace sample from a ^{14}C-CF stock was directly injected into a septum-sealed LSV containing 15 mL MP cocktail. A second such injection was made to the combustion chamber. Combustion, oxygen purge, and collection of the products in alkaline cocktail (15 mL [illegible] monoethanolamine) were carried out exactly as in [illegible] analysis for $^{14}CH_4$. Comparison of dpm values in the two vials (after subtraction of background levels) indicated 92.2 percent [illegible]ery.

4. Analysis of $^{14}CO_2$ and Nonstrippable ^{14}C-Residues

a. Method

The starting point for $^{14}CO_2$ analysis was an anaerobic degradation system which had previously been used in $^{14}CH_4$ analysis. Thus, it possessed a pH > 10.5. A 20 mL aliquot of the alkaline contents was poured into a stripping chamber fitted with a septum-sealed side arm (Figure 5). The top of the chamber was then sealed to the bottom via a ground-glass joint (\$24/40), a liberal amount of high-vacuum grease, and a pair of springs. A porous-glass diffuser (ASTM porosity C, 25-50 micrometers) projected through the top to the bottom of the chamber. A flow of high-purity nitrogen gas passed through the diffuser into the 20 mL sample; from the stripping chamber through a 10 cm length of plastic tubing which contained 0.25 g Tenax™; through a second diffuser identical to the first; and into a $^{14}CO_2$ trap consisting of 20 mL of 0.5 N NaOH. The purpose of the Tenax™ was to sorb organic forms of ^{14}C which might otherwise end up in the NaOH trap and contribute positive error to the analysis of $^{14}CO_2$. The quantity of NaOH used in the trap was designed to provide greater than three times the equivalents of OH^- as there were moles of C_T in a 20 mL sample being stripped.

After introducing the alkaline sample, sealing the stripping chamber, and initiating a N_2 flow of 50-60 mL/min, a 0.4 mL volume of glacial acetic acid was injected through the side arm, dropping the pH to 4.4. (Low pH values -- e.g., pH ~ 2 -- apparently caused significant hydrolysis of organic solids in the sludge, resulting in severe foaming problems in the stripping chamber. If a strong acid such as HCl or H_2SO_4 were used, one could very easily overdose and generate this low-pH problem. On the other hand, acetic acid, with its pK_a of about 4.7, provided sufficient buffering in the region of desirable pH values to eliminate concern.)

Stripping was carried out for a minimum of 30 minutes. After this period, the contents of the NaOH trap were poured into a 25 mL volumetric flask. Approximately 3 mL of washings from trap and diffuser were added, and the flask was diluted to the 25 mL mark with distilled water, stoppered, and shaken. A 2 mL volume of the diluted

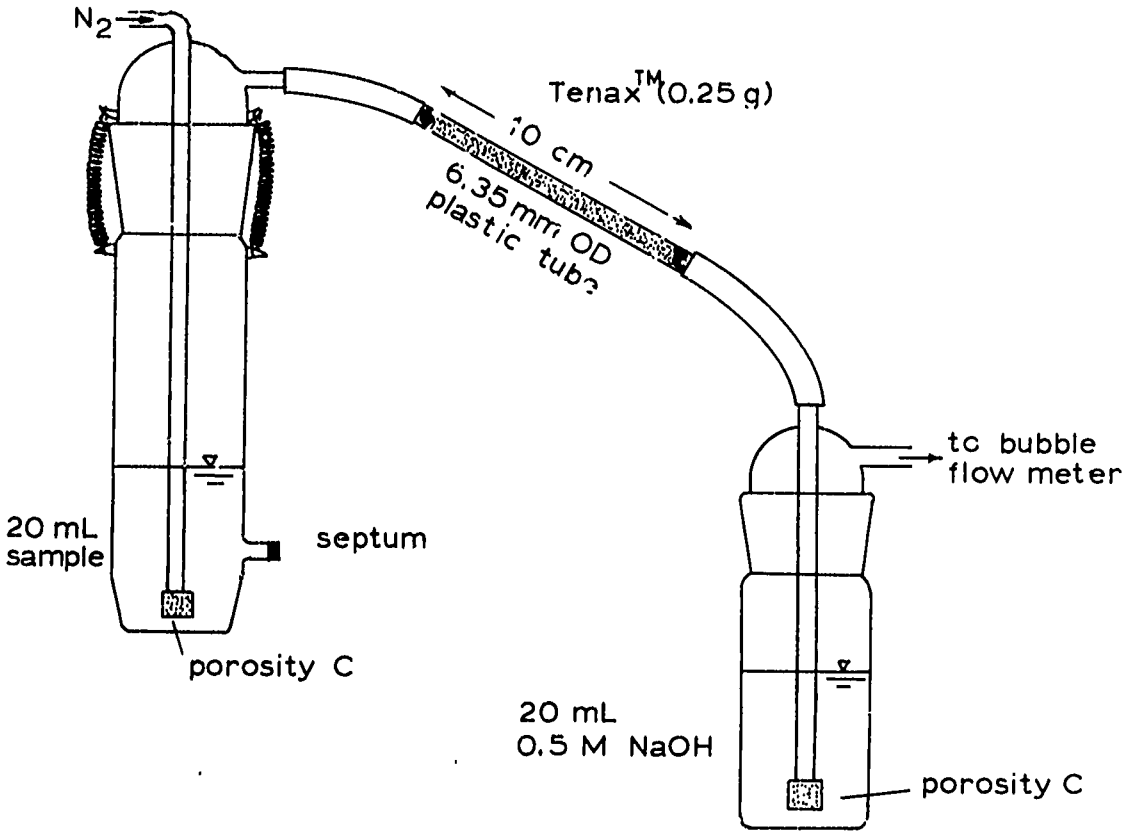

Figure 5. Stripping Chamber.

trap was added to a LSV containing 15 mL MP cocktail for presumptive analysis of $^{14}CO_2$. As a check, 10 mL of the diluted trap was added to a 25 mL glass (Corex™) centrifuge tube, to which 0.1 g $BaCl_2 \cdot 2H_2O$ was added as a nearly quantitative precipitant of $CO_3^=$. The tube was capped, shaken, and centrifuged at 3500 rpm for 10 minutes (IEC centrifuge model DPR-6000). A 2 mL volume of the centrate was added to a second LSV containing 15 mL MP cocktail. Comparison of the dpm values from the two vials served to check the ability of the method to discriminate properly against noncarbonate sources of ^{14}C. In no instance did the nonprecipitable dpm in the NaOH trap exceed 3 percent of the total; dpm values observed in the NaOH trap were almost exclusively derived from carbonates. The total dpm attributable to forms of $^{14}CO_2$ in an anaerobic degradation system were thus calculated as follows:

$$^{14}CO_2 \text{ dpm} = (dpm_{meas} - \text{blank})(25/2)(101.5/20) \quad (6)$$

In Equation (4), the dpm_{meas} refers to the value obtained from the first LSV (i.e., without prior precipitation of carbonates by Ba); the blank value is obtained by LSC of a vial containing 15 mL MP cocktail and 2 mL of a 0.5 N NaOH solution; the 101.5 value refers to the liquid volume of the anaerobic system from which the 20 sample was obtained for stripping analysis.

The residue remaining in the purge chamber after the 30 minutes of stripping was also analyzed. A wide-bore, 1.00 mL graduated pipet was used to obtain a 0.5 mL sample of the stripping chamber's contents. This aliquot was directly added to a LSV containing 15 mL of MP cocktail. In addition, the remaining liquid in the chamber was added to a 50 mL, stainless-steel centrifuge tube and spun at 14,000 rpm for 10 minutes (Sorvall centifuge, model RC2-B Superspeed). A 0.5 mL volume of the centrate was added to a second LSV, identical to the first. The dpm value observed in the LSV containing the whole sample allowed the calculation of the total "nonstrippable ^{14}C" in the original, anaerobic degradation system. Use of the second LSV allowed determination of the proportions of this nonstrippable residue associated with solid and soluble fractions.

b. Evaluation of Method

Concerns with the possible collection of noncarbonate sources of ^{14}C in the NaOH trap were adequately addressed through the use of a barium chloride precipitation check. There were, however, other concerns.

To determine whether or not a 30-minute purging period was sufficient to strip all carbonates from a 20 mL sample, an experiment was performed in which a fresh NaOH trap was installed after an initial 30 minutes of stripping, followed by another 30-minute purge. Subsequent analyses of both traps indicated that only 1.2 percent of the total dpm was associated with the trap from the second 30 minutes of purging.

The efficiency with which a single NaOH trap collects $^{14}CO_2$ was evaluated by addition of a second trap, placed in series with the first. After purging a 20 mL sample from one of the anaerobic degradation systems for 30 minutes, the two traps were analyzed by LSC. Less than 1 percent of the total, collected dpm were contributed by the second NaOH trap.

5. Liquid Scintillation Counting

^{14}C analyses were performed using a Beckman model LS 9800 liquid scintillation counter. Appropriate background corrections were always incorporated into the determination of dpm in sample vials (by counting vials containing MP cocktail, MP cocktail + NaOH, or MP cocktail + monoethanolamine). Counts per minute (cpm) were converted to disintegrations per minute (dpm) through use of a quench curve based on sample H#. The quench curve was generated from a set of quenched ^{14}C standards covering an H# range from 0 to 364. All samples had H#s within the range of this quench curve. The highest degree of quenching was encountered with residue analysis (H# ~ 200), where 0.5 mL of suspension was directly added to MP cocktail. Other sample types had H#s from 75 to 125.

Chemiluminescence was encountered with cocktail containing monoethanolamine. Analysis of the energy spectrum indicated that the dpm associated with this chemiluminescence was of low energy; 90 percent could be eliminated from accumulated counts by setting the channel threshold level at 200. Consequently, a counting window between 200 and 670 was used to assay for ^{14}C. (A mere 2 percent of the ^{14}C spectrum from an unquenched standard was below this energy window).

Samples were generally counted until the random counting error in cpm was less than 2 percent (at the 95 percent confidence level). However, some low-level background samples and cocktail blanks were only counted to a 5-9 percent precision (with 95 percent confidence), due to a 20-minute ceiling placed on the allowable duration of counting.

G. ANALYSIS OF CH_4, CO_2, AND N_2 BY GAS CHROMATOGRAPHY WITH TCD

A gas chromatograph with a thermal-conductivity detector (Sigma 2000, Perkin-Elmer) was employed for the analysis of methane, carbon dioxide, and nitrogen gases in the seed digester, in headspaces of anaerobic degradation bottles, and in the purge streams employed during various anaerobic transfer techniques. GC conditions were as follows: injector and detector temperatures, 200°C; column oven temperature, 150°C; carrier (He) flow, 60 mL/min (split between the two channels of the TCD); column, 3.05-meter by 3.2 mm stainless-steel, packed with 100/120 Carbosieve S-II (Supelco, Inc.); filament current, 150 mA. Sample size was 1.0 mL in all cases. The detection limit was approximately 5×10^{-5} atm for all three compounds (based on the injection of a 1.0 mL gas sample at 1 atm total pressure). Calibration curves were prepared by injecting a series of different volumes of each gas; linear regressions of the resulting data (GC peak area vs. amount of compound) yielded calibration factors with coefficients of determination (r^2 values) exceeding 0.999 in all instances.

H. GAS PRODUCTION

In a few degradation studies, the cumulative gas productions from serum bottles were monitored over time. A volume displacement method

was used. A 500 mL aspirator bottle was used as a reservoir, containing about 400 mL of an aqueous, saturated NaCl solution, acidified with 5 percent H_2SO_4, and with 10 mg methyl orange added to provide color for ease in reading fluid levels. A latex tube led from the bottom of the reservoir to the bottom of a 10 mL graduated pipet which was vertically mounted on a ring stand. The top of the pipet was connected by a short piece of latex tubing to a three-way, plastic stopcock. One fitting of this stopcock was left free; the remaining fitting was attached to a 0.6-meter length of latex tubing which terminated in a 5 cm side-port needle. A platform was attached to a second ringstand to support the reservoir such that the levels of displacement fluid in both reservoir and pipet were equal and at the zero mark on the pipet.

It would have been preferable to perform gas production measurements entirely within a room held at 35°C (the incubation temperature). Since facilities did not permit this, bottles had to be removed from the incubator and equilibrated to ambient temperature prior to measurement. Gas production (since previous measurement) was determined by manipulating the stopcock so that the passage between the needle and pipet was open; the needle was inserted through the bottle's septum; the reservoir was lowered such that its fluid level equalled that of the displaced fluid level in the pipet (ensuring that the displacement volume reflected atmospheric pressure); the displacement was recorded; the reservoir was raised to its normal position on the platform; the stopcock was manipulated to allow open passage between the needle, the pipet, and the ambient atmosphere (and thus, the displacement fluid returned to the zero mark, now at the same level as that of the reservoir fluid, while the accumulated pressure in the serum bottle was vented to the atmosphere); and the needle was withdrawn from the serum bottle.

Each day that gas production volumes were measured, the ambient temperature and pressure were recorded, allowing data to be expressed in milliliters at standard temperature (273°K) and pressure (760 mm Hg).

The equation used to correct measured displacement volume (V_m, ml) to standard conditions is:

$$ml(STP) = (P_m/T_m)(21.125 + 0.3592\ V_m) - 21.125\ (P_o/T_o) \qquad (7)$$

where

P_m = atmospheric pressure when V_m was measured (mm Hg);

T_m = temperature when V_m was measured (^oK);

P_o = atmospheric pressure when bottle was previously vented (i.e., at last reading) (mm Hg);

T_o = temperature when bottle was previously vented (^oK).

The derivation of this equation is based upon the ideal gas law and assumes a bottle headspace of 58.8 mL.

I. OXIDATION-REDUCTION POTENTIAL (ORP)

ORP (E_H) values were measured with an epoxy ORP electrode with platinum band (Cole-Parmer Inst. Co.), in conjunction with a pH meter with expanded-scale capability (Corning Model 12). The ORP electrode had an internal Ag/AgCl (saturated) reference. Thus, observed mV readings were corrected to represent estimates of E_H by the addition of +198.8 mV (the E_H value of the reference at 25°C). The diameter of this combination electrode was small enough to fit through the opening of a 158.8 mL serum bottle. ORP measurements were made by removing the seal from a serum bottle, inserting the electrode, and gently swirling the contents of the bottle while observing the mV scale of the pH meter. All measurements were made only after bottles had been equilibrated to ~25°C, since ORP values are temperature-dependent, and a value of E_H for the reference at 35° was not available.

J. MISCELLANEOUS ANALYTICAL METHODS

The following parameters were measured in accordance with procedures outlined in Standard Methods (Reference 33): pH (Section 423); alkalinity (Section 403, with a pH end-point of 4.0); total solids (TS) and volatile solids (VS) (Section 209 G).

SECTION IV

ANAEROBIC DEGRADATION OF TETRACHLOROETHYLENE AND TRICHLOROETHYLENE

A. TETRACHLOROETHYLENE

Figure 6 depicts results from batch, anaerobic incubation of tetrachloroethylene (PCE) in seeded samples, autoclaved-seed controls (ASC), and water controls (WC). Each bottle received a 640-nanomole initial dose of PCE. This is equivalent to a nominal concentration of about 1.06 mg/L. However, since the added PCE partitioned itself between the gaseous and solution phases, the actual, added, aqueous concentration would have been approximately 0.64 mg/L. Seeded-sample and WC systems were prepared in duplicate. Data in Figure 6 represent mean values of these duplicates, in some instances; however, the usual monitoring technique was to alternate sampling from duplicates (i.e., one day's sample was obtained from the first of the pair; the next day's sample would come from the second member of that duplicate pair). This explains the saw-tooth shape occasionally apparent in plots.

Results indicate significant sorption of PCE to solids contributed by the seed; this is assumed to be the cause of the observed difference between the curves for the WC and the ASC. Such a difference was evident from the beginning of incubation. The slow decline in mass of PCE from the WC systems is taken as evidence of leakage. Despite the apparent leakage, its rate is sufficiently low so as to cause no problems with data interpretation. It is obvious that in the systems containing viable, anaerobic organisms (described by lines with labels unmodified by parenthetic suffix in Figure 6), PCE was completely and stoichiometrically converted to TCE. This occurred by Day 40. Note that the quantity of TCE formed by that time (approximately 410 nanomoles) appears to exceed the quantity of PCE present in the ASC. However, examination of later data from the study of TCE (Figure 7) provides the explanation: TCE does not appreciably sorb onto the seed sludge solids, whereas PCE does; therefore, when all PCE is converted to TCE, the TCE level should reach approximately that of PCE in the WC (which it did), rather than that of the ASC.

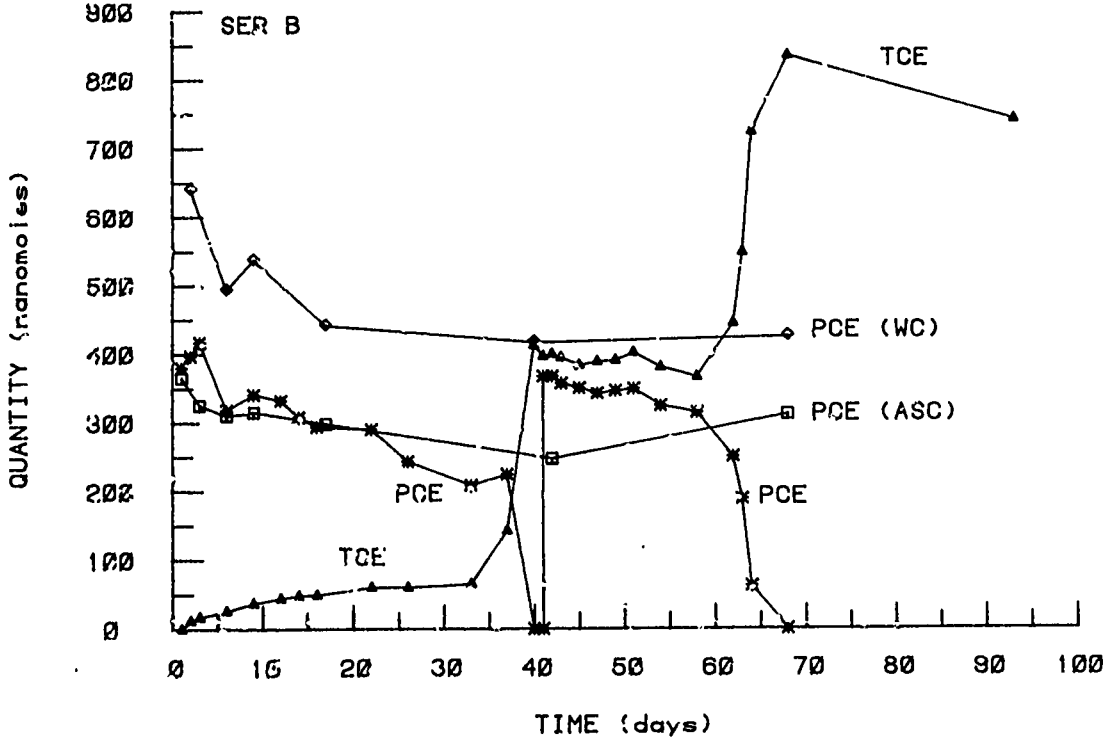

Figure 6. Extended Anaerobic Incubation of Tetrachloroethylene (PCE).

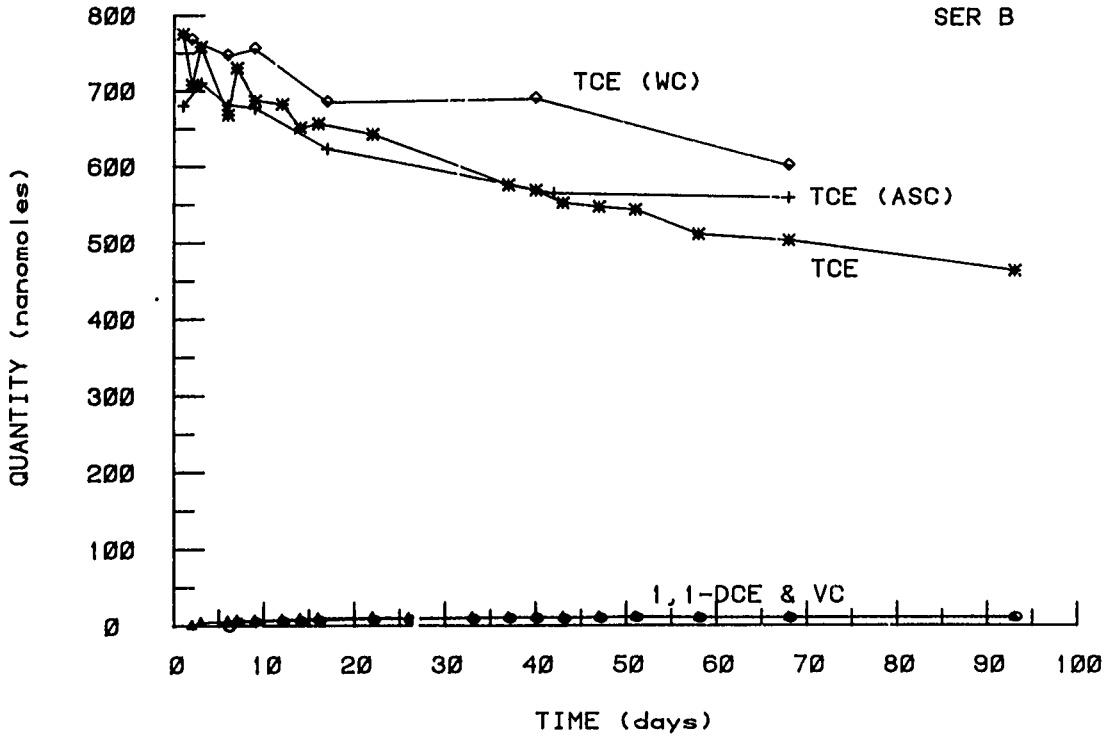

Figure 7. Extended Anaerobic Incubation of Trichloroethylene (TCE).

On Day 41, one of the seeded samples was respiked with another 640 nanomoles of PCE (Figure 6). After a lag of approximately 20 days, the PCE was again quantitatively converted to TCE over the ensuing 3 or 4 days. Following this, the TCE level slowly declined through the end of the period of study. However, only traces (relative to the TCE level) of expected TCE-degradation intermediates and products were detected. For example, the sum of both cis- and trans-1,2-DCE never exceeded 20 nanomoles; neither 1,1-DCE nor VC was evident. Therefore, the fate of the TCE lost from samples beyond Day 70 is not known. Slow leakage cannot be excluded as a plausible mechanism.

B. TRICHLOROETHYLENE

Figure 7 depicts results from anaerobic incubation of systems originally dosed with 760 nanomoles of TCE. This amount corresponds to a nominal, added aqueous concentration of about 1.00 mg/L. When partitioning to the gas phase is considered, the initial solution concentration from such a dose would have been approximately 0.74 mg/L. As with the PCE study, WC and seeded-sample systems were prepared and monitored in duplicate, but an alternating pattern of sampling was generally employed.

Sorption of TCE to seed solids appears to have been minimal. There is virtually no difference between measured TCE amounts in the different types of systems over the first 20 days of incubation -- a period sufficient to have allowed detection of sorption, if it had been significant. All that is evident from Figure 7 is a slow decline in TCE levels from all three types of systems, with perhaps the seeded samples exhibiting the greatest rate of its disappearance. Some leakage was inevitably involved in the observed disappearance. And of course, the sampling procedure itself removed some of the TCE (but calculation shows this to be insignificant: a mere 1.3 nanomoles of TCE would be removed in sampling 0.5 mL of headspace from a bottle containing 500 nanomoles total TCE).

Small amounts of expected intermediates and products of TCE degradation were detected. The compounds, and their maximum, observed quantities in seeded samples were: 1,1-DCE (11 nmol); cis-1,2-DCE (27

nmol); and VC (12 nmol). Considering the quantity of TCE added and the quantity which disappeared from seeded samples (approximately 300 nmol), these levels of expected intermediates and products may appear too small to support a claim that any significant portion of the 300 nmol loss of TCE from seeded samples was the result of biological degradation via such intermediates. On the other hand, a metabolic intermediate would not necessarily achieve a high concentration if its subsequent conversion to other, undetected products was rapid. It is also possible that sorption of intermediates to seed sludge solids was significantly greater than for any of the five volatile solutes whose sorption was investigated -- and ruled either insignificant or tolerable -- in preliminary studies (described in Section III-E-1). Quantities would have been significantly underestimated by the headspace GC technique for any compound which sorbed significantly. Support for the position that at least some of the observed loss of TCE from seeded samples was the result of biological activity comes from the fact that none of these suspected intermediates was detected in WC or ASC systems.

C. DISCUSSION

Previous studies by other investigators (References 27 and 28) have indicated that PCE can be anaerobically degraded -- via TCE, DCE isomers, and VC -- to carbon dioxide. In this present study, degradation appears to have ceased with the production of quantitative amounts of TCE, and only trace levels of the 1,2-DCEs. Experiments in which the starting compound was TCE showed only its slight conversion to DCEs and VC. The reason remains unknown. One can conjecture that a cometabolite or suitable primary substrate was missing; that a required growth factor was not supplied; or that a suitable organism (or set of organisms) was lacking in the seed.

Results suggest that the microbially mediated reductive dechlorination of PCE is an inducible process. Approximately 40 days were required to effect conversion of PCE to TCE when the inoculum was first exposed to PCE; and nearly all of the conversion occurred over the final 3 days of that period (Figure 6). After then "resting" in the absence of PCE for 1-2 days, the seeded system required approximately 20

days to acclimate to a subsequent dose of additional PCE. Again, the bulk of this second conversion occurred within just 3 days after this second acclimation period. The first acclimation period could be claimed to represent the effects of population growth (i.e., the growth of a suitable population of PCE-dechlorinating organisms) as well as induction of the requisite machinery for reductive dechlorination. However, the occurrence of a second lag period upon respiking (and after the system had been without PCE for less than 2 days) suggests the action of an inducible system for the conversion of PCE. With the disappearance of the originally added PCE, the dechlorinating microorganisms involved in the process probably recycled the now excess, unneeded enzymatic machinery into other, more useful materials -- or energy. In essence, they simply "forgot" how to convert PCE. That the acclimation or lag period was nearly halved the second time around may merely reflect an elevation in the population of PCE-dechlorinating organisms resulting from the first period of acclimation/conversion, or the fact that the organisms may not have completely eliminated the previously assembled metabolic machinery necessary for the task.

Based solely upon these results, it is not possible to determine whether or not microorganisms derive usable energy via the reductive dechlorination of PCE. The source of reducing power (i.e, the electron donor) has also not been identified. Though starved for several days prior to use as an inoculum, the mixed liquor of the seed digester no doubt remained a sufficiently rich source of complex substrates to fulfill the cometabolic or primary substrate requirements of organisms dechlorinating a mere 1 mg/L PCE. The implication in finding that a metabolic process is inducible is that the process is carried out for a purpose. In the case at hand, it may be merely that the mediating organisms derive some unknown, selective advantage from the dechlorination process.

SECTION V

ANAEROBIC DEGRADATION OF 1,1,1-TRICHLOROETHANE

A. EXTENDED INCUBATION

An 850-nanomole dose of 1,1,1-trichloroethane (1,1,1-TCA) was injected to seeded-sample, ASC, and WC systems, with subsequent incubation for 93 days. The amount added corresponds to a nominal, aqueous concentration of about 1.13 mg/L. When partitioning to the gas phase is considered, the initial solution concentration from such a dose would have been approximately 0.71 mg/L. WC and seeded-sample systems were prepared in duplicate, but sampling was generally alternated between duplicate bottles on successive days. Results are shown in Figure 8. Plots representing data from WC and ASC systems are labeled with compound names, followed by "(WC)" and "(ASC)," respectively. Plots of data from seeded samples are labeled without a parenthetical suffix.

Leakage was apparently significant, as is evident from the substantial decline of 1,1,1-TCA in WC systems. However, a portion of this loss reflects abiotic, dehydrohalogenation of 1,1,1-TCA to 1,1-DCE, which reached a level of 60 nmol in the WC systems. Sorption to seed solids was not appreciable, since the initially recovered levels of 1,1,1-TCA in ASC and WC systems were approximately equal.

Most noteworthy is the rapid conversion of 1,1,1-TCA to 1,1-DCA (followed by a much slower production of EtCl) in the viable systems, with essentially no acclimation required. Within the first 6 days of incubation, virtually all of the added 1,1,1-TCA had disappeared. In its place were 335 nmol 1,1-DCA. This accounts for less than 40 percent of the added 1,1,1-TCA. The fate of the other 515 nmol of added 1,1,1-TCA is unknown. Certainly leakage was significant, but not sufficient to explain the loss. After 93 days of incubation, the 1,1-DCA level had dropped to 221 nmol, with EtCl rising to a level of 56 nmol.

If 1,1-DCA were a rapidly utilized intermediate in the metabolism of 1,1,1-TCA, its observed peak level would not be expected to equal that of the added 1,1,1-TCA. However, 1,1-DCA remained at relatively constant levels long after the complete disappearance of 1,1,1-TCA.

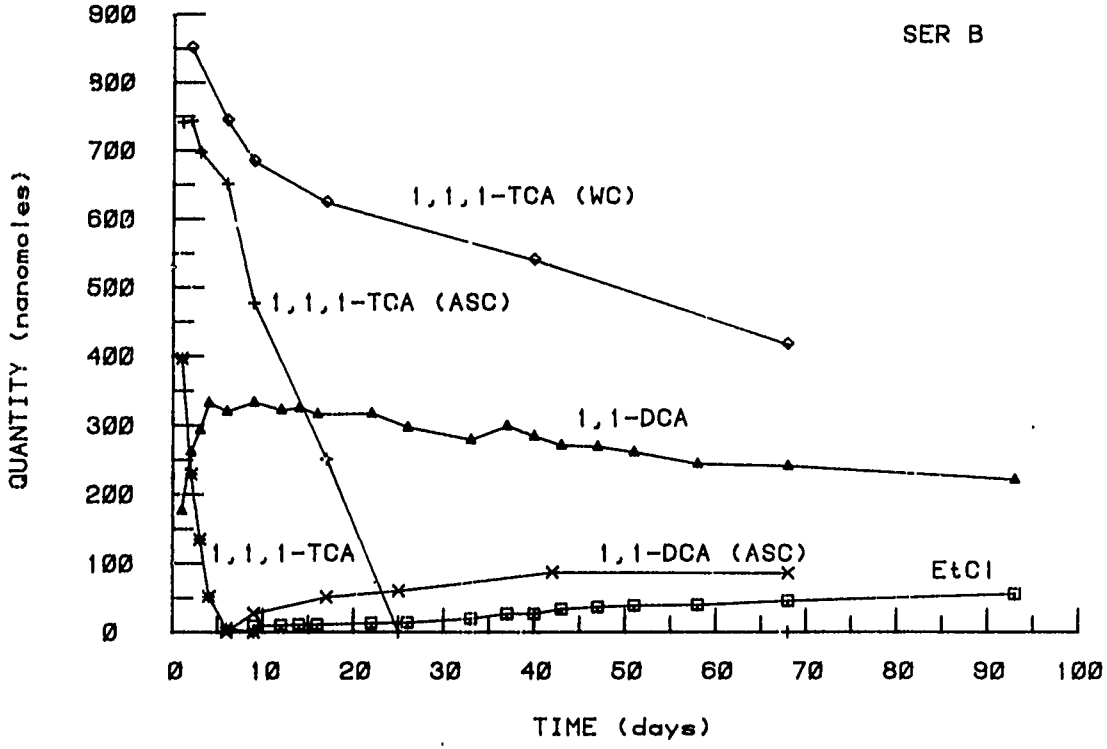

Figure 8. Extended Anaerobic Incubation of 1,1,1-Trichloroethane (1,1,1-TCA).

Therefore, the maximum level of 1,1-DCA achieved can be expected to reflect the stoichiometry of 1,1,1-TCA utilization. It is possible that the lack of apparent molar conservation between added 1,1,1-TCA and observed levels of 1,1-DCA is caused by the oxidation of one portion of the 1,1,1-TCA to undetected intermediates or products, providing the reducing power for the reductive dechlorination of another portion of 1,1,1-TCA to 1,1-DCA. An alternative explanation may be sorption of 1,1-DCA to seed solids. No sorption studies were conducted with this compound. If sorption occurred, then analysis via headspace GC would have underestimated the total amount of 1,1-DCA present.

There was a substantial rate of loss of 1,1,1-TCA from the ASC system, with at least some conversion to 1,1-DCA (Figure 8). Within 25 days, all added 1,1,1-TCA had disappeared from the autoclaved-seed control. The maximum, observed quantity of 1,1-DCA was only 87 nmol; no EtCl was detected. The implication is that reductive dechlorination of 1,1,1-TCA to 1,1-DCA may occur abiotically under reducing conditions. The difference between rates observed with the viable systems, compared to that of the ASC system, may merely be the result of differences in chemical environment between viable and autoclaved-seed systems. However, pH and ORP values were similar in the two types of systems (Table 4).

Autoclaving the seed for 30 minutes at 121°C may be insufficient to inactivate all enzymes, resulting in some activity in the ASC system. Contamination after autoclaving cannot be ruled out either. Additional studies were performed to address issues of abiotic dechlorination under reducing conditions and the effectiveness of a 30-minute period of autoclaving.

B. ABIOTIC STUDIES WITH REDUCING AGENTS

A series of 158.8 mL serum bottles was prepared in which various chemical reducing agents were present in 100 mL of oxygen-free, buffered water. The water was prepared as follows: 10 grams of $NaHCO_3$ was added to 2 liters of distilled water in a flask (equivalent alkalinity = 3,000 mg/L as $CaCO_3$); this solution was boiled for 10 minutes; and a cannula was inserted to purge the contents continuously with a CO_2/N_2

mixture (30/70 mole percent ratio), while the solution cooled to room temperature. Preparation of each serum bottle consisted of the following sequence: the empty bottle was purged of air via the cannula system; the reducing agent was added in solid form; 100 mL of the buffered, oxygen-free water was anaerobically transferred to the bottle (using the system described in Section III-D-2); and the bottle was sealed with a Teflon™-lined, rubber septum and aluminum crimp cap. After all bottles had been thus prepared, each was dosed with 1325 nmol 1,1,1-TCA via a 0.2 mL injection of oxygen-free water which had been saturated with 1,1,1-TCA. Each was then vigorously shaken and transferred to an incubator at 35°C. Each different type of system was prepared in triplicate, to provide some bottles for measurement of ORP; after measurement, such bottles were discarded.

Eight types of systems were studied. All had 100 mL liquid volumes: (1) aerobic, distilled water; (2) buffered, oxygen-free water (BOFW); (3) 500 mg/L dithioerythritol in BOFW; (4) 500 mg/L $FeCl_2 \cdot xH_2O$ in BOFW; (5) 500 mg/L $Na_2S \cdot 9H_2O$ in BOFW; (6) 500 mg/L $SnCl_2 \cdot 2H_2O$ in BOFW; (7) 100 mL of autoclaved, anaerobic inoculum (prepared in the usual manner of an ASC system), with autoclaving for 30 minutes at 121°C prior to injection of the 1,1,1-TCA; and (8) a second ASC, autoclaved for 60 minutes at 121°C. These last two types of systems are termed "ASC(30)" and "ASC(60)," respectively, in subsequent tables and figures. Their purpose was to investigate the effectiveness of the 30-minute period of autoclaving normally used in all other phases of this research.

Results are shown in Figure 9. All types of systems demonstrated similar rates of decrease in 1,1,1-TCA level. Judging from the difference between the ASC systems and the others after 1 day of incubation, sorption of 1,1,1-TCA to seed solids appears to have been significant, whereas it was not in the previous series of studies (Figure 8). Because of periodic additions of fresh, municipal digester sludge to the seed digester, the character of the seed varied among the different series of studies.

Both ASC systems exhibited virtually the same rate and extent of disappearance of 1,1,1-TCA. And both accumulated relatively low levels of 1,1-DCA (approximately 60 to 90 nmol after 26 days). The implication

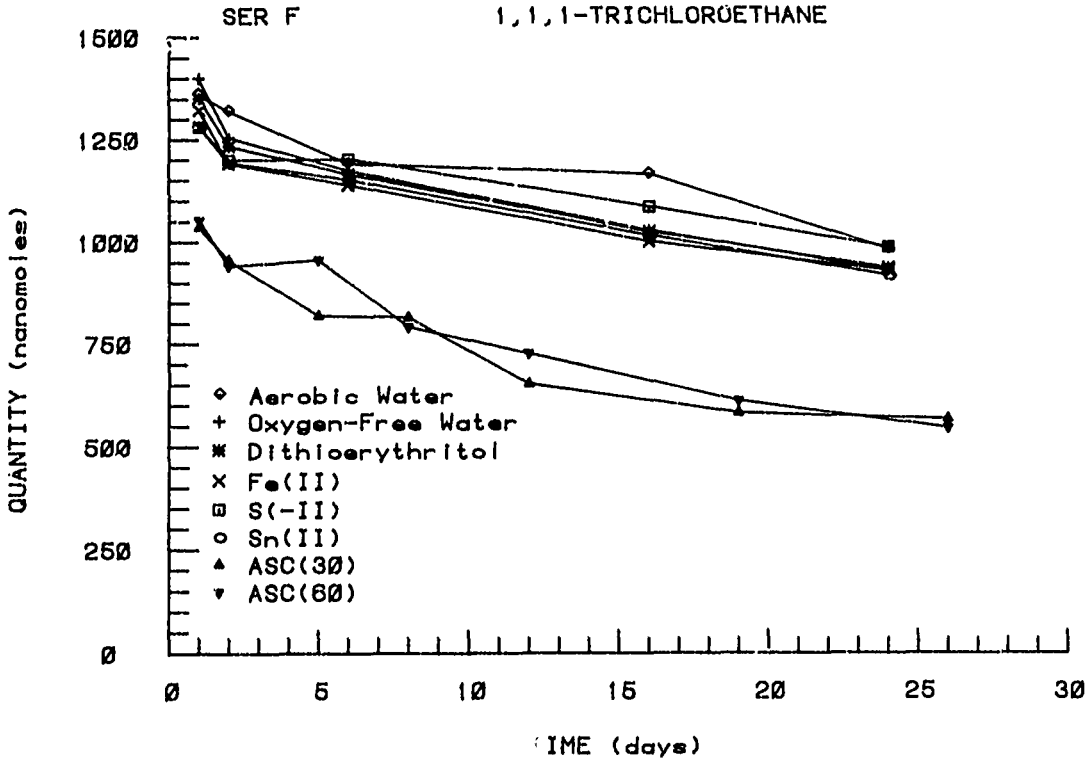

Figure 9. Abiotic Incubation of 1,1,1-Trichloroethane in the Presence of Various Chemical Reductants.

is that 30 minutes is a sufficient period in which to achieve whatever degree of organism or enzyme destruction is possible with autoclaving at 121°C. The more rapid disappearance of 1,1,1-TCA in the ASC depicted in Figure 8 is probably the result of contamination.

Measured values of ORP are contained in Table 6. A significant range is encompassed by these studies, yet the data in Figure 9 do not indicate significant differences in rate of 1,1,1-TCA decrease among the different systems. Furthermore, 1,1-DCA was not detected in any of systems (with the exception of the ASCs); on the contrary, they accumulated small (typically 40-60 nmol) quantities of 1,1-DCE, regardless of ORP.

C. REPETITIVE ADDITIONS OF 1,1,1-TRICHLOROETHANE

Figure 10 summarizes results from a study in which the response of an inoculated system to repetitive additions of 1,1,1-TCA was monitored. By observing the pattern of response, it was hoped that something could be inferred regarding the role of 1,1,1-TCA degradation in the overall metabolism of the mediating microorganism(s). The added 1,1,1-TCA was repeatedly utilized, with concommitant production of 1,1-DCA in a stoichiometric proportion equal to approximately 40 percent of the 1,1,1-TCA consumed. This is in accordance with earlier findings (Figure 8).

Figure 11 presents these same 1,1,1-TCA-degradation data on a larger scale, along with data from the WC. If one is careful to compare the repetitive decay curves only over portions where their initial and final 1,1,1-TCA levels coincide, then it is evident that the kinetics of degradation did not change appreciably over the course of seven, sequential additions. For example, approximately 2.5 days were required for the 1,1,1-TCA level to decrease from 1200 to 100 nmol in all cases. The rate of 1,1,1-TCA utilization appears to be in some manner proportional to its concentration; each repeating curve exhibits a negative slope whose absolute magnitude diminishes as the quantity of 1,1,1-TCA decreases. (But one should resist the temptation to subject these data to quantitative, kinetic analysis. The systems were quiescently

TABLE 6. MEASURED ORP VALUES IN ABIOTIC STUDIES[a]

Type of System	Time of Incubation (days)	EH (mV)
Aerobic, Distilled Water	2	+439
	24	+450
Buffered, O_2-Free Water (BOFW)	2	+399
	24	+379
Dithioerythritol in BOFW (500 mg/L)	2	-141
	24	-175
$FeCl_2 \cdot xH_2O$ in BOFW (500 mg/L)	2	- 76
	24	- 56
$Na_2S \cdot 9H_2O$ in BOFW (500 mg/L)	2	-211
	24	-211
$SnCl_2 \cdot 2H_2O$ in BOFW (500 mg/L)	2	- 76
	24	- 49
Autoclaved-Seed Control [ASC(30]	2	-142
	24	-138

[a] Values measured at 25°C.

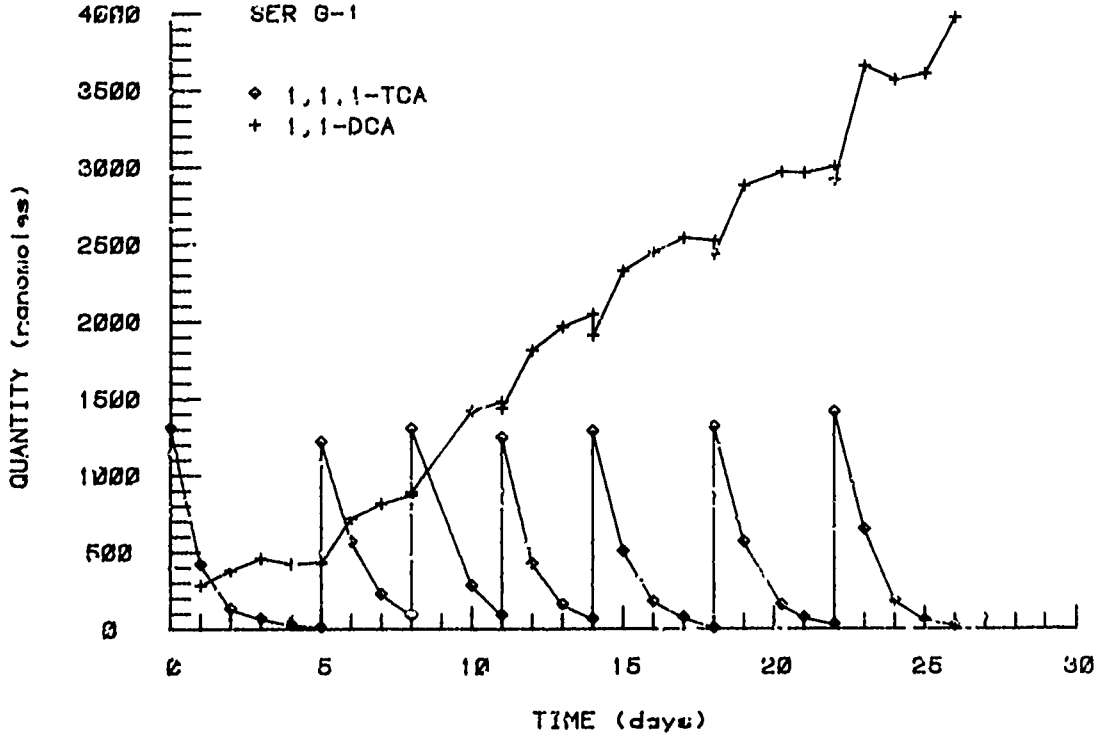

Figure 10. Repetitive-Addition Studies With 1,1,1-Trichloroethane (WC Results Omitted).

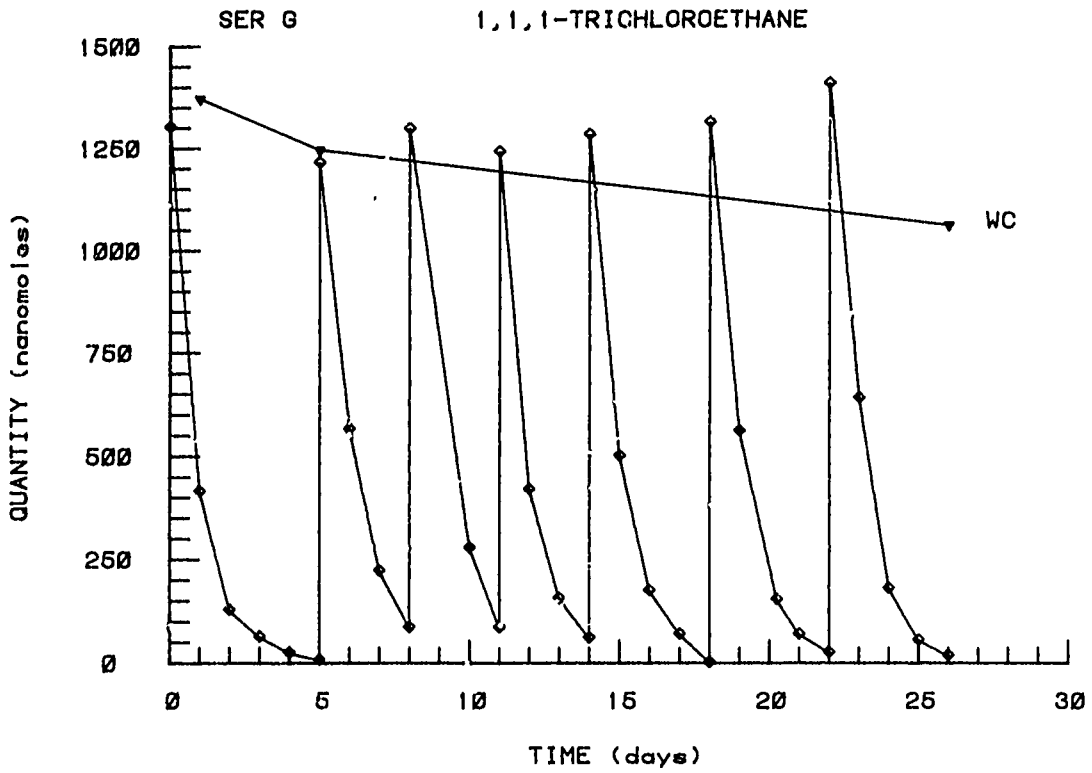

Figure 11. Repetitive-Addition Studies With 1,1,1-Trichloroethane (1,1-DCA Data Omitted).

incubated. Mixing occurred sporadically -- only during periods of headspace analysis.)

D. DISCUSSION

Microbial conversion of 1,1,1-TCA under anaerobic conditions proceeded without acclimation. Results suggest that a major portion of the compound (at least 40 percent -- possibly much more) was reductively dechlorinated to 1,1-DCA, which accumulated in this study. The lack of an acclimation period suggests that 1,1,1-TCA is processed by a constituitive enzyme system (or at least one which was already functioning prior to the first addition of 1,1,1-TCA to this inoculum). The unchanged rate of its utilization during a sequence of repetitive additions implies that 1,1,1-TCA does not serve as a growth substrate for the organisms involved. In other words, its utilization may be a metabolic accident, of little or no benefit to the mediating microorganisms. However, it is possible that their population was sufficiently high in the inoculum that the amount of growth occurring on the total, 9.0-micromole quantity of 1,1,1-TCA added in the seven, repetitive injections was negligible by comparison, resulting in no apparent increase in utilization rate over the course of the study.

Experiments performed with a variety of chemical reducing agents over a range of ORP values suggests that abiotic, reductive dechlorination of 1,1,1-TCA may not occur at ambient temperatures and neutral pH. However, ORP is a very imperfect measure of the reducing nature of an environment. The ability of a particular abiotic environment to bring about reductive dechlorination of 1,1,1-TCA is no doubt more related to the presence of some specific reductant(s), than it is to the indiscriminate measure provided by a platinum electrode. Therefore, all that can be justifiably concluded is that the specific reductants employed in this study do not bring about the reductive dechlorination of 1,1,1-TCA to 1,1-DCA. Minor levels of 1,1-DCA were detected in ASC systems; this suggests that suitable, unidentified, abiotic reductants may exist. However, it is equally likely that the observed levels of 1,1-DCA were the result of microbial contamination, or that some enzymatic activity

survived autoclaving. In any event, the rates of dechlorination observed in ASC systems were -- with one exception -- negligible, by comparison to those observed in seeded systems.

SECTION VI

ANAEROBIC DEGRADATION OF CHLOROFORM

A. EXTENDED INCUBATION

A 4.85-micromole dose of chloroform (CF) was injected to seeded-sample, ASC, and WC systems, with subsequent incubation for 93 days. The added amount corresponds to a nominal, aqueous concentration of about 5.8 mg/L. When partitioning to the gas phase is considered, the initial solution concentration from such a dose would have been approximately 5.1 mg/L. WC and seeded-sample systems were prepared in duplicate, but sampling was generally alternated between members of a duplicate pair on successive days. Results are shown in Figure 12. Neither sorption nor leakage of CF was apparent in this phase of study, as is evident from examination of WC and ASC data.

CF was rapidly degraded, without apparent lag. Within 9 days, all added CF had disappeared from the seeded systems. In its place were approximately 1.50 micromoles of DCM — an amount roughly equal to 31 percent of the added CF — and inconsequential (0.05 micromole) levels of CM. This DCM remained at a near constant level for another 21 days, exhibiting only a very slight rate of decline. From t =30 days onward, the rate of DCM utilization increased somewhat, though remained low in comparison to that initially observed in the case of CF. CM was observed over this period of increased DCM utilization; the CM level peaked at 0.41 micromoles (t = 45 days), decreasing steadily afterwards.

Since the peak level of DCM was maintained long after CF had disappeared, then one can justifiably conclude that DCM was not a rapidly utilized intermediate in this study; therefore, the peak level achieved (in relation to that of the added CF) can be taken as representing the stoichiometric proportion of DCM formed from CF by the microbial system employed. Later experiments with DCM indicate that this compound is unlikely to have sorbed significantly (which would have caused underestimation of DCM levels); therefore, the 31 percent observed conversion of CF to DCM is probably an accurate reflection of stoichiometric proportion. The fate of the other 69 percent of added CF is unknown,

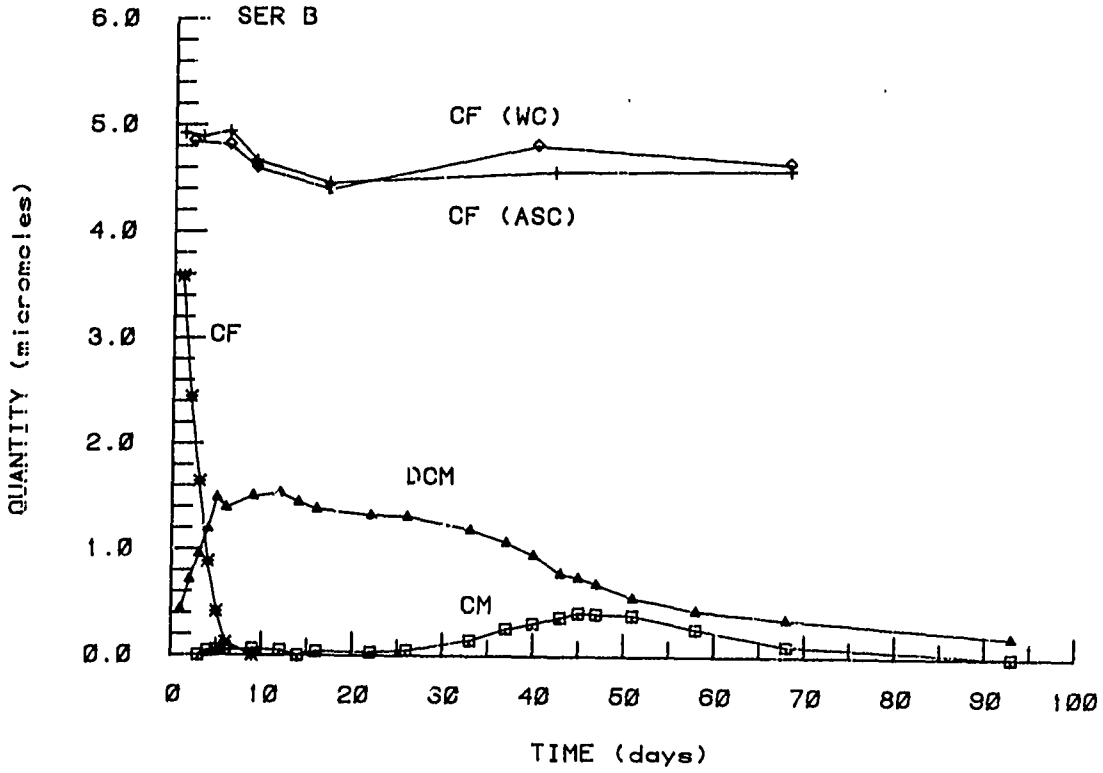

Figure 12. Extended Anaerobic Incubation of Chloroform (CF) (MeSH Data Omitted).

based solely upon this phase of study. Relatively enormous quantities of MeSH were formed in seeded systems (Figure 13); however, the absolute level of accumulated MeSH far exceeded the quantity of CF added, suggesting that MeSH is not a direct product of CF degradation. Later studies with ^{14}C-CF -- discussed in Section VI-D -- support this conclusion.

Zinder and Brock (References 37 and 38) have presented evidence that MeSH is an intermediate in the formation of methane from the terminal methiol group of methionine. Their work also suggests that CF inhibits methane production from methionine, causing accumulation of MeSH. Data presented in Figure 13 support this hypothesis, and tend to implicate DCM as an additional inhibitor of MeSH utilization. When the level of DCM was eventually reduced to below about 1 micromole, MeSH was rapidly consumed. Further studies were initiated to examine the utilization of MeSH and the effects of CF and DCM upon it.

B. ANAEROBIC DEGRADATION OF METHANETHIOL

As a peripheral part of this research, the effect of CF and DCM on the anaerobic utilization of MeSH was investigated. Seeded serum bottles were prepared in the usual manner; some contained seed only; some contained seed + 7 micromoles MeSH; some contained seed + 7 micromoles MeSH + 5 micromoles CF; and some contained seed + 7 micromoles MeSH + 9.5 micromoles DCM. Figure 14 shows the effects of the chlorinated compounds on utilization of MeSH. MeSH was rapidly degraded in the absence of CF or DCM. In bottles containing DCM, the MeSH level remained unchanged over the 18 days of incubation. In bottles with CF present, the MeSH level actually increased over the first 14 days of incubation, followed by a dramatic decline. Figure 15 shows the cause: the added CF was depleted by Day 14, alleviating the inhibition to MeSH utilization. The DCM formed from CF utilization apparently never achieved an inhibitory level.

Gas production data from this phase of study are shown in Figure 16, and serve to illustrate the levels of inhibition. The greatest production of gas was achieved by the system containing MeSH in the absence of chlorinated organics. This system appears to have surpassed

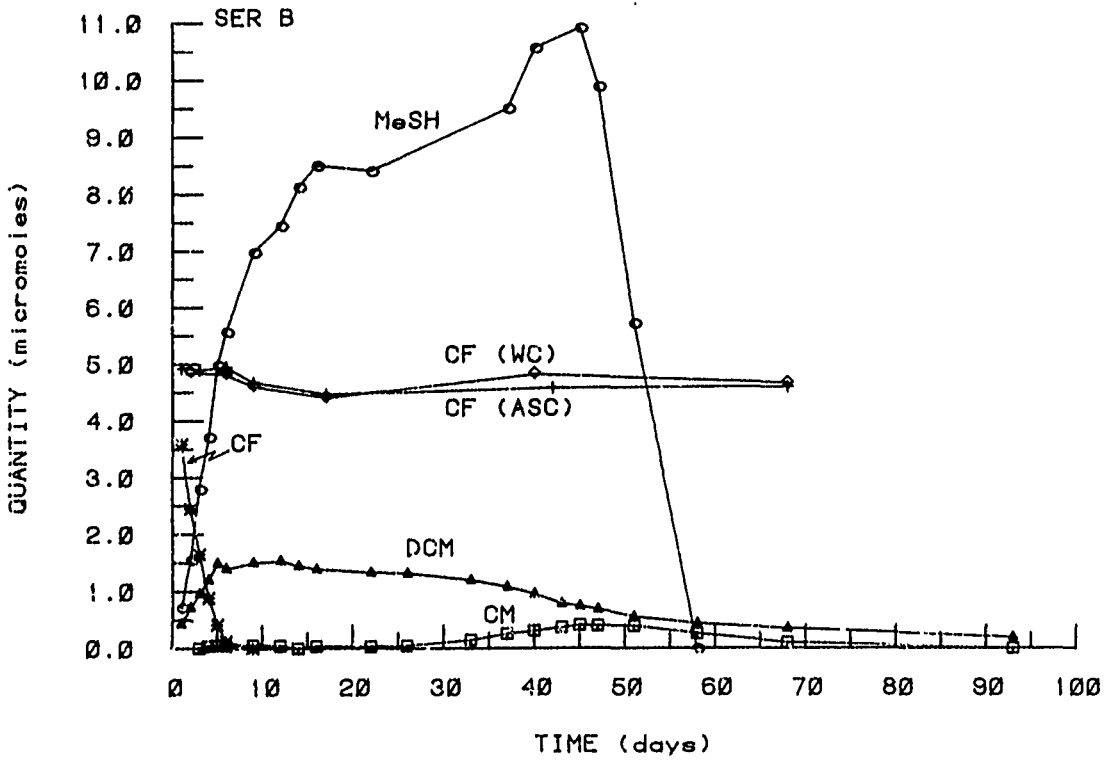

Figure 13. Extended Anaerobic Incubation of Chloroform.

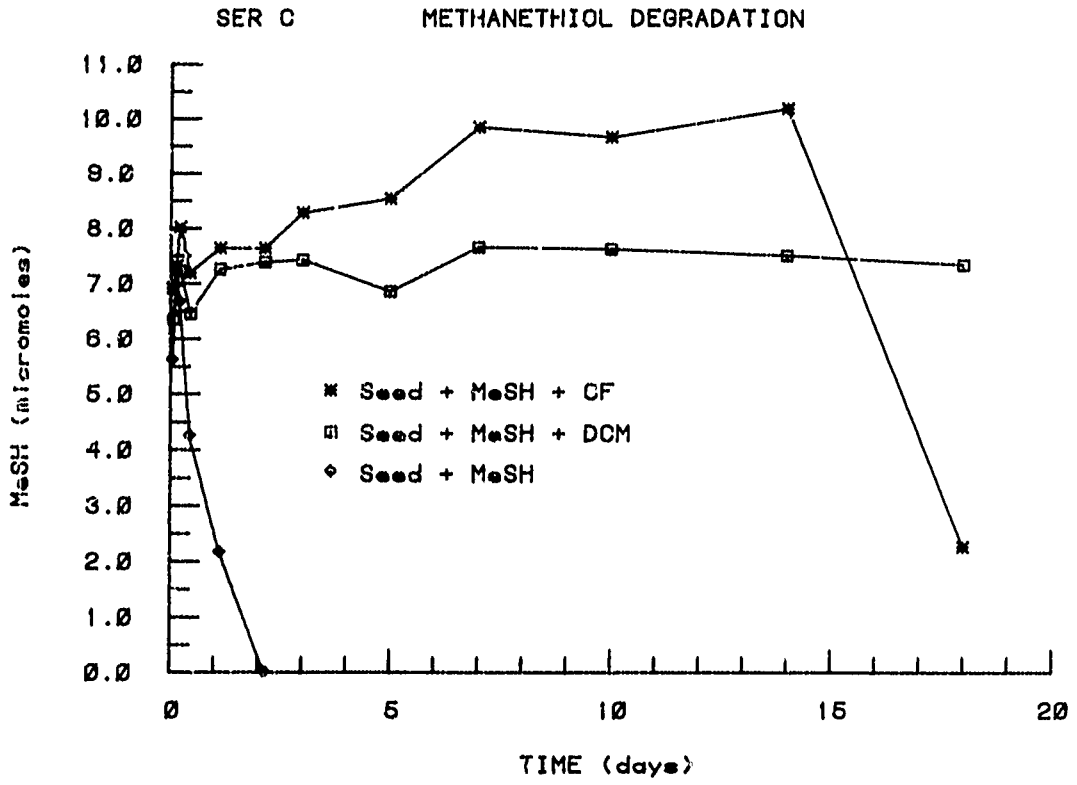

Figure 14. Effects of Chloroform or Dichloromethane on the Anaerobic Degradation of Methanethiol.

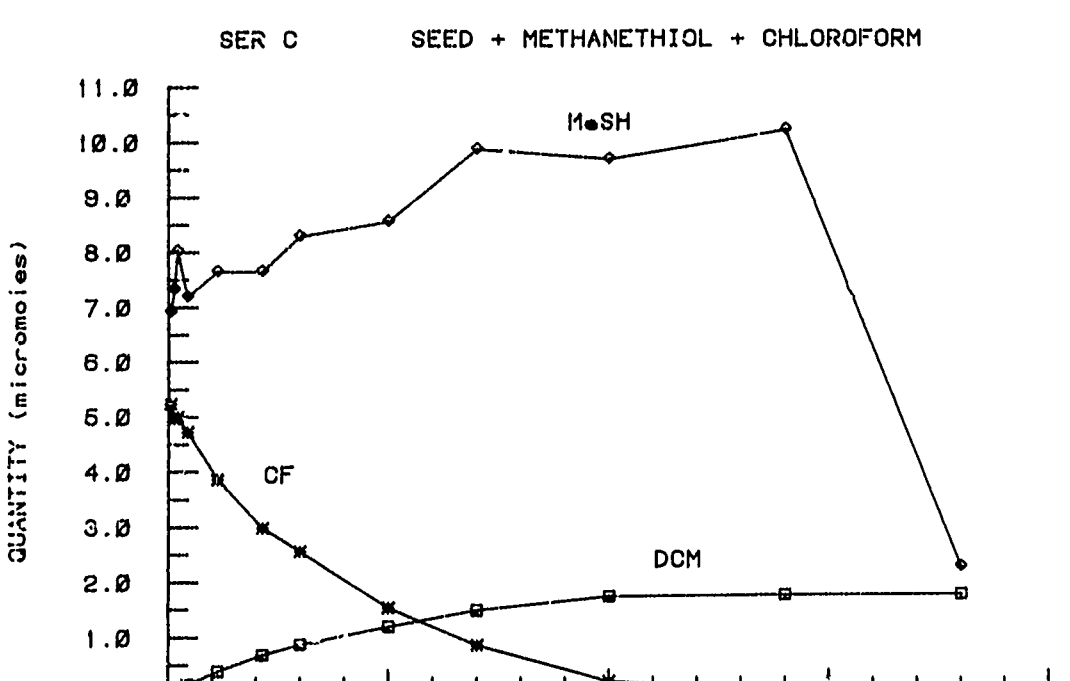

Figure 15. Chloroform (CF), Dichloromethane (DCM), and Methanethiol (MeSH) Levels Resulting From Anaerobic Incubation of MeSH in the Presence of CF.

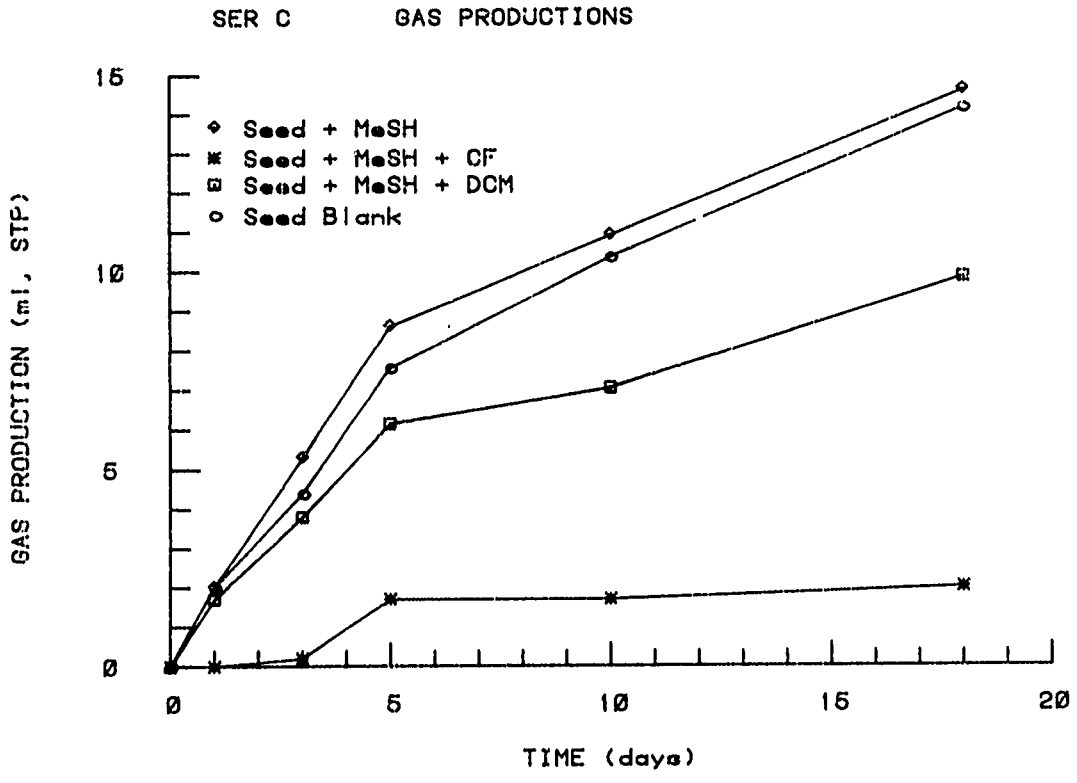

Figure 16. Cumulative Gas Productions From MeSH Studies.

the seed blank in gas production. However, the differences are not statistically significant; furthermore, the volume of methane to be expected from the complete conversion of 7 micromoles of MeSH is only about 0.16 mL (STP) -- a volume small enough to be lost in the random errors of gas monitoring. The system containing MeSH and DCM seems to have suffered some degree of inhibition. But the most noticeable inhibition was exhibited by the CF-dosed bottle

These results support the findings of Zinder and Brock, regarding the inhibitory effects of CF on MeSH utilization by methanogenic systems. Additionally, data presented in Figures 13 and 14 suggest that DCM can exert the same effects, though higher concentrations appear to be required to achieve inhibition comparable to that observed with CF. Results here suggest that DCM levels below 1-2 micromoles (i.e., 10-20 micromoles per liter) are not absolutely inhibitory to utilization of MeSH; whereas, CF was inhibitory at any detectable concentration.

C. THE EFFECTS OF CHLOROFORM LEVEL ON ITS DEGRADATION RATE

A series of serum bottles was prepared in which three different levels of CF were initially added. (Along with the addition of stable CF, a small amount of radiolabeled CF was also added, because these same bottles served as the basis for a radiotracer study which is described in the next subsection of this report. The use of ^{14}C-CF is only incidental to the phase of study described in this present subsection). The added quantities of CF used were 16.0, 4.75, and 1.60 micromoles. Bottles of each type were prepared in triplicate: one was used only for gas-production measurements; the other two were monitored routinely via headspace analysis, with one bottle eventually sacrificed for analysis of radiolabeled products.

Figure 17 shows results from these systems. Since the bottles were incubated quiescently and were shaken sporadically (i.e., only during headspace analyses), quantitative kinetic analysis of the data was not performed. However, one can conclude that the rate of CF utilization appears to be proportional to the remaining CF concentration (to some positive exponent).

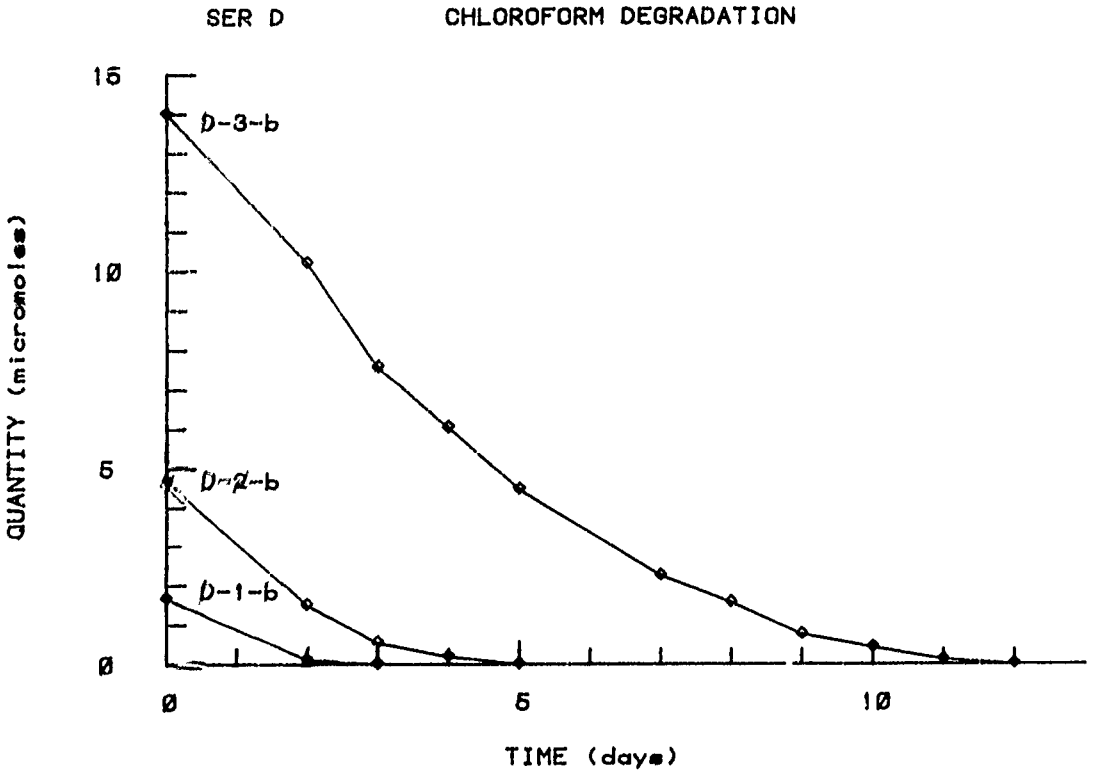

Figure 17. Effect of Chloroform Level on its Degradation.

Figure 18 presents gas production data from this phase of study. Gas data from the bottle originally dosed with 4.75 micromoles CF are not shown, because this bottle was sacrificed for other experimental purposes soon after it was prepared. The gas data indicate the inhibitory effects of CF on anaerobic, microbial activity in general. The higher CF dose resulted in only slightly greater inhibition.

D. STUDIES WITH RADIOLABELED CHLOROFORM

Along with the addition of different amounts of stable CF, each of the bottles described in the previous subsection was originally prepared with the addition of 2.0 mL from a stock ^{14}C-CF solution containing 1.264×10^6 dpm/mL. Thus, each bottle should have received 2.523×10^6 dpm. When routine headspace monitoring indicated that all of the added CF had disappeared, bottles were then subjected to extensive analysis to determine the fate of the added, radiolabeled CF. Three bottles, representing each of the three initial levels of CF, were analysed. To put the ^{14}C analyses in proper context, the levels of CF, DCM, and MeSH in these three bottles — based on headspace analyses — are depicted in Figures 19, 20, and 21. The composition of a bottle at the time of ^{14}C analysis is that of the last day shown in the respective Figure.

Results of ^{14}C analyses are summarized in Table 7. The amount of ^{14}C that remained in the residue after stripping of $^{14}CO_2$ was not assayed in the case of the bottle that had been dosed at the lowest CF level. For the other two bottles, agreement between the total CF dpm added and the sum of dpm values in the assayed categories after incubation is quite good (91 and 97 percent).

The results from these radiolabeled CF studies indicate that from 28 to 34 percent of added CF is converted to DCM. This is in agreement with results described in Section VI-A. The fate of the other 66 to 70 percent of added CF is now clearer: most of it is converted to CO_2. From 32 to 44 percent of added ^{14}C-CF was recovered as $^{14}CO_2$. There also appears to be a trend of decreasing $^{14}CO_2$ with increasing initial CF level. However, it must be pointed out that the higher the initial CF level, the longer was the incubation period prior to ^{14}C analysis. The trend — if real — could be more related to time than dose.

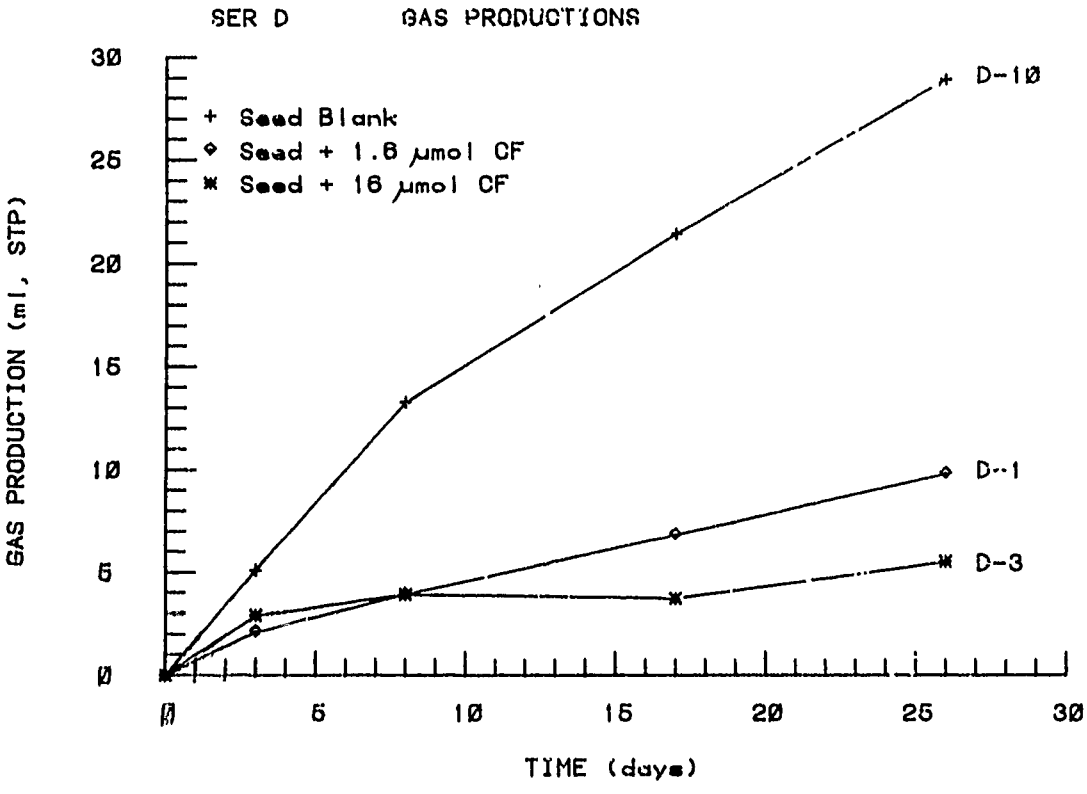

Figure 18. Effect of Chloroform Level on Cumulative Gas Production.

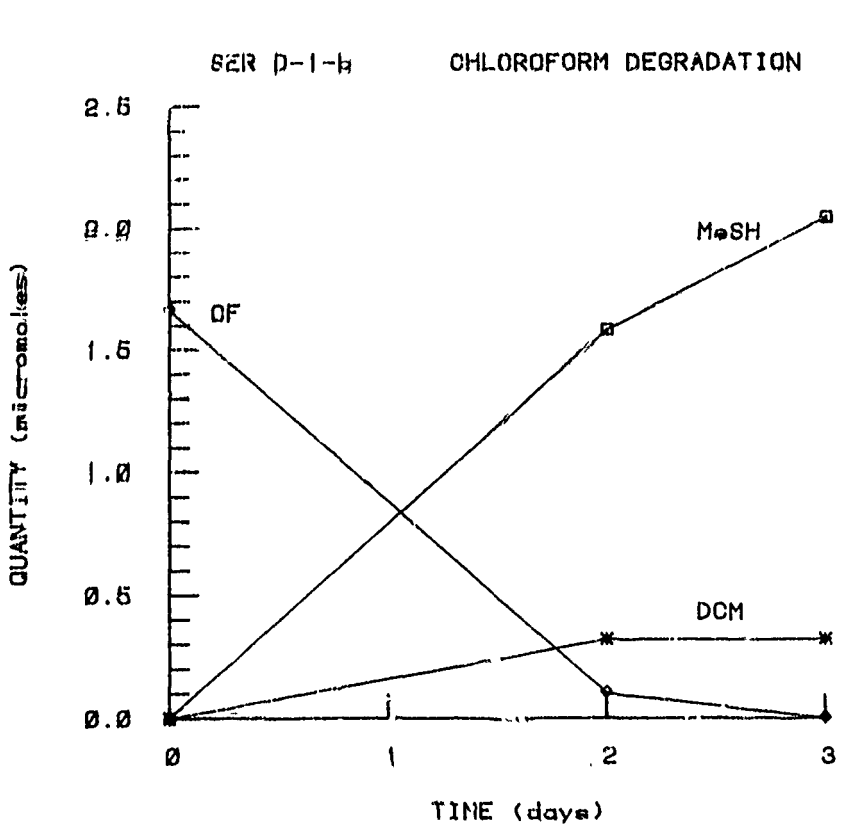

Figure 19. Incubation of Bottle D-1-b to Point of ^{14}C Analysis.

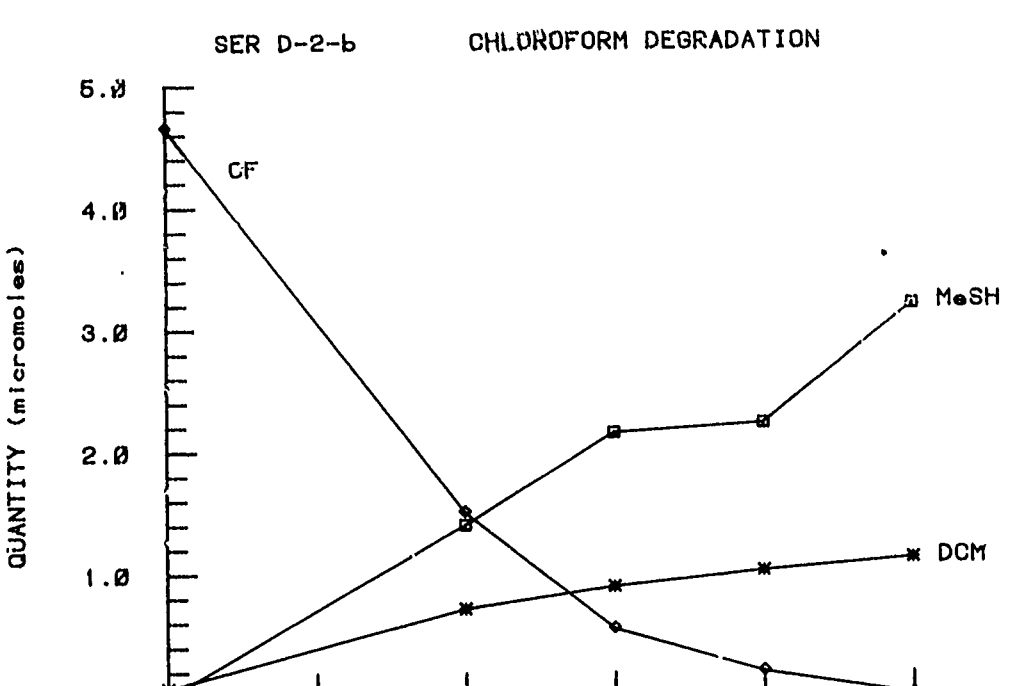

Figure 20. Incubation of Bottle D-2-b to Point of ^{14}C Analysis.

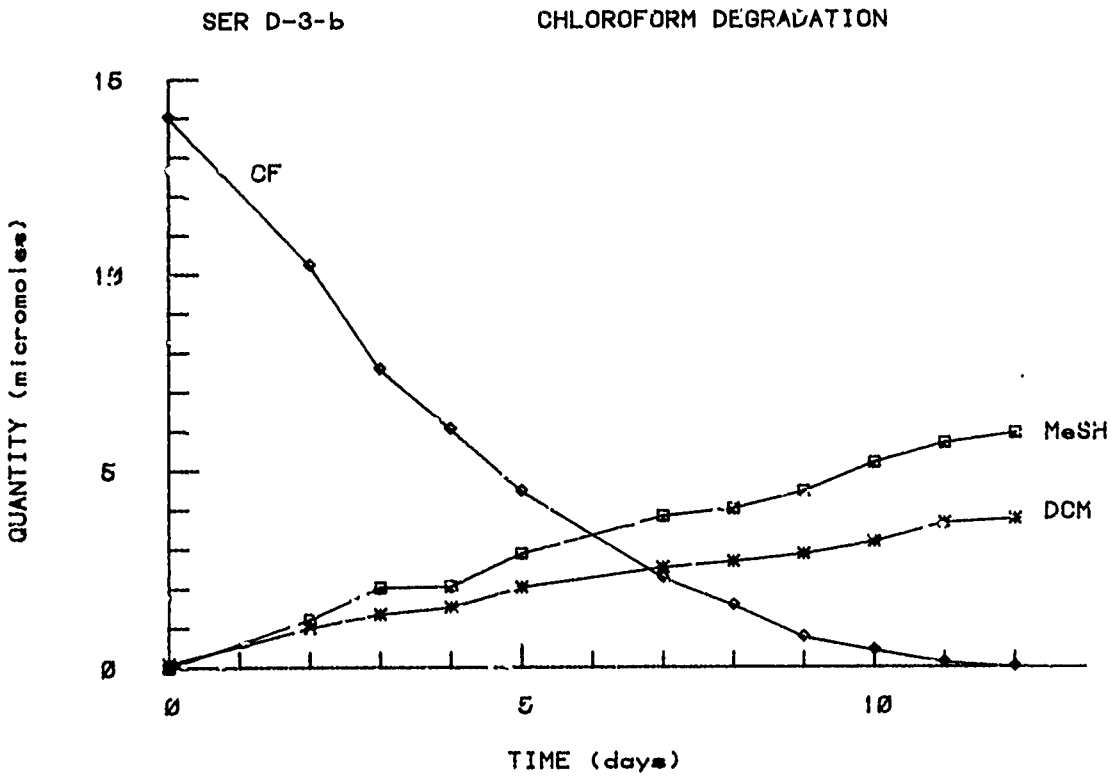

Figure 21. Incubation or Bottle D-3-b to Point of ^{14}C Analysis.

TABLE 7. DISTRIBUTION OF ^{14}C AFTER DEGRADATION OF LABELED CF

Bottle	CF Added (micromoles)	Fraction ID	dpm	% of Added dpm[a]
D-1-b (t=3 d)	1.60	CM	0	0
		DCM	861,000	34.1
		MeSH	25,400	1.0
		CH_4	15,900	0.6
		CO_2	1,100,000	43.5
		Total	2,002,300	
D-2-b (t=5 d)	4.75	CM	0	0
		DCM	756,000	29.9
		MeSH	41,700	1.6
		CH_4	2,350	0.1
		CO_2	1,020,000	40.3
		Nonstrippable Residue	640,000[b]	25.3
		Total	2,460,050	97.3
D-3-b (t=12 d)	16	CM	0	0
		DCM	699,000	27.7
		MeSH	59,800	2.4
		CH_4	0	0
		CO_2	812,000	32.1
		Nonstrippable Residue	732,000[c]	29.0
		Total	2,302,800	91.1

[a] 2.528×10^6 dpm ^{14}C-CF added.

[b] Of this total, 54 percent was associated with the soluble phase.

[c] Of this total, 42 percent was associated with the soluble phase.

Perhaps the stoichiometric proportion of $^{14}CO_2$ produced from ^{14}C-CF was constant at all CF levels, but that systems incubated for longer periods exhibited greater assimilation of $^{14}CO_2$.

It is clear from the inconsequential levels of radiolabeled MeSH recovered, and the substantial levels of total MeSH present in the three bottles (Figures 19, 20, and 21), that MeSH is not a direct product of CF utilization. Likewise, the data demonstrate that CF is not converted to CH_4 to any significant extent. The small quantities detected were likely the result of $^{14}CO_2$ reduction after its formation from ^{14}C-CF. The existing pool of carbonates was substantial (on the order of 7 mM), providing many orders of magnitude dilution of the $^{14}CO_2$ released from ^{14}C-CF degradation.

From 25 to 29 percent of the added ^{14}C-CF appeared in the nonstrippable residue; and of this, approximately 40 to 50 percent was soluble. The definitions of soluble and solid used here were operationally based upon centrifugation after stripping at pH 4.4. It seemed possible that the low-pH conditions may have solubilized ^{14}C material which would not otherwise have been solubilized at pH 7. An experiment was performed to investigate this possibility — and to evaluate other criteria for defining "soluble" and "solid" fractions.

A sample of bottle D-2-C (a replicate of the second system described in Figure 20 and Table 7) was fractionated at pH 7.1 via preliminary centrifugation (3500 rpm, 10 minutes), followed by vacuum filtration through a 0.22-micrometer membrane. Samples of both this filtrate and the original, whole sample were then acidified to pH 4.4 with glacial acetic acid and purged for 30 minutes in the fashion of a usual, $^{14}CO_2$ assay. The nonstrippable residues from each were analyzed by LSC: the soluble residue contained 41 percent of the dpm contained in the total residue. This is a lower number than the value measured when "soluble" was defined at pH 4.4 (54 percent of total dpm was declared "soluble"); however, the use of a 0.22-micrometer membrane filter in the later procedure probably had more to do with this "discrepancy" than did the pH at separation. In any event, the results do not differ remarkably. It remains valid to conclude that from 25 to 29 percent of the added ^{14}C-CF ended up as a nonstrippable residue; and that from 40 to 50 percent of this residue consisted of soluble matter.

E. REPETITIVE ADDITIONS OF CHLOROFORM

Figure 22 presents results from a study in which the response of an inoculated system to repetitive additions of CF was monitored. It is apparent that the added CF was repeatedly utilized -- without lag. In every case, the rate of utilization exhibited the CF-concentration dependence seen previously (i.e., see Figure 17). However, if one is careful to compare the repetitive decay curves only over portions where their initial and final CF levels coincide, it is apparent that the rate of utilization decreased with each successive addition of CF. For example, the time required to effect a decrease in CF from 2.25 to 0.5 micromoles exhibited the following pattern with the first five successive additions: 1.5 days, 3.0 days, 4.0 days, 4.4 days, and 5.0 days.

Several possible explanations for the decrease in utilization rates were investigated. Perhaps CF utilization was only secondary to consumption of some other, primary, growth substrate -- a substrate which was being depleted in this study. As one means of testing this hypothesis, 1.0 mL of a buffered glucose/acetate mixture (20 g/L glucose + 10 g/L glacial acetic acid + 5 g/L $NaHCO_3$ in oxygen-free water) was injected along with the CF at t = 25 days. The resulting concentrations of glucose and acetic acid in the serum bottle were 200 mg/L and 100 mg/L, respectively. Following this, the time required to achieve the 1.75-micromole reduction in CF (from 2.25 to 0.5 micromoles) was 4.6 days -- not a significant improvement.

Another possible explanation for the decreasing CF utilization kinetics is that DCM had accumulated to inhibitory levels after repeated production from CF. DCM levels are shown in Figure 23, which is an expanded-scale version of Figure 22. At t = 30 days, the bottle was opened (under a 30 percent CO_2/70 percent N_2 atmosphere induced via a cannula purge), purged for 10 minutes to strip most of the DCM, sealed, and then injected with additional CF stock. The intention was to observe the kinetics of CF utilization in the absence of appreciable DCM. Unfortunately, an error was made: DCM was added along with the CF. After waiting 4 days for the CF level to drop, the intended

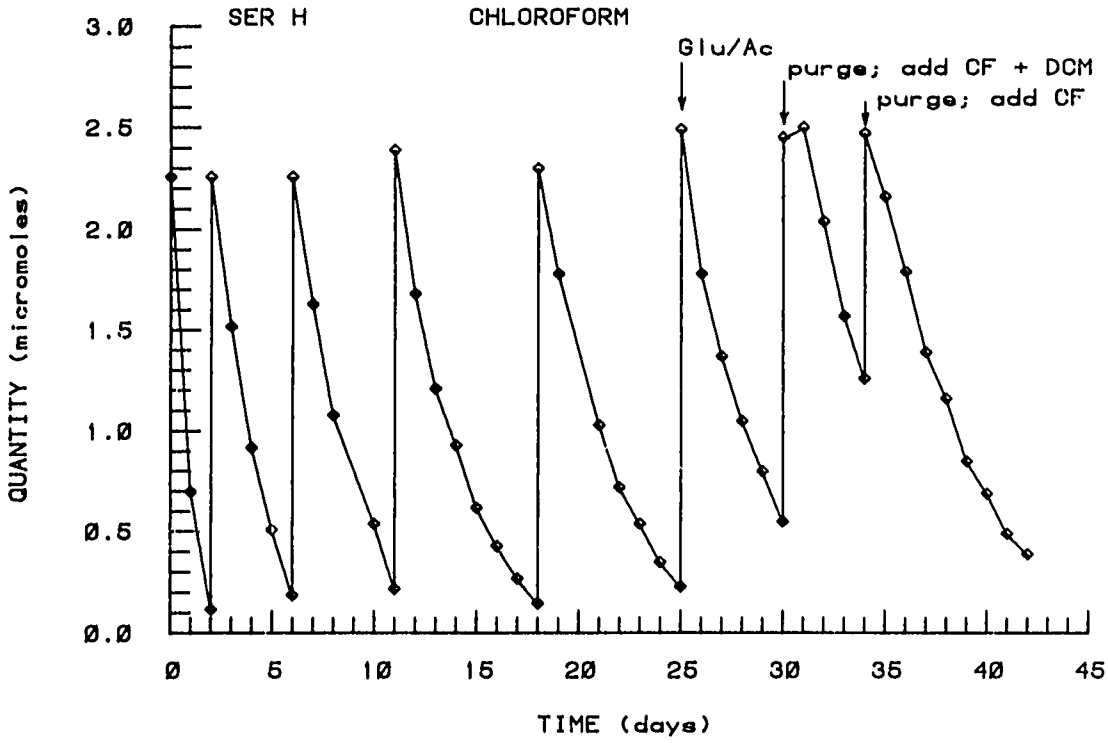

Figure 22. Repetitive-Addition Studies With Chloroform (DCM Data Omitted).

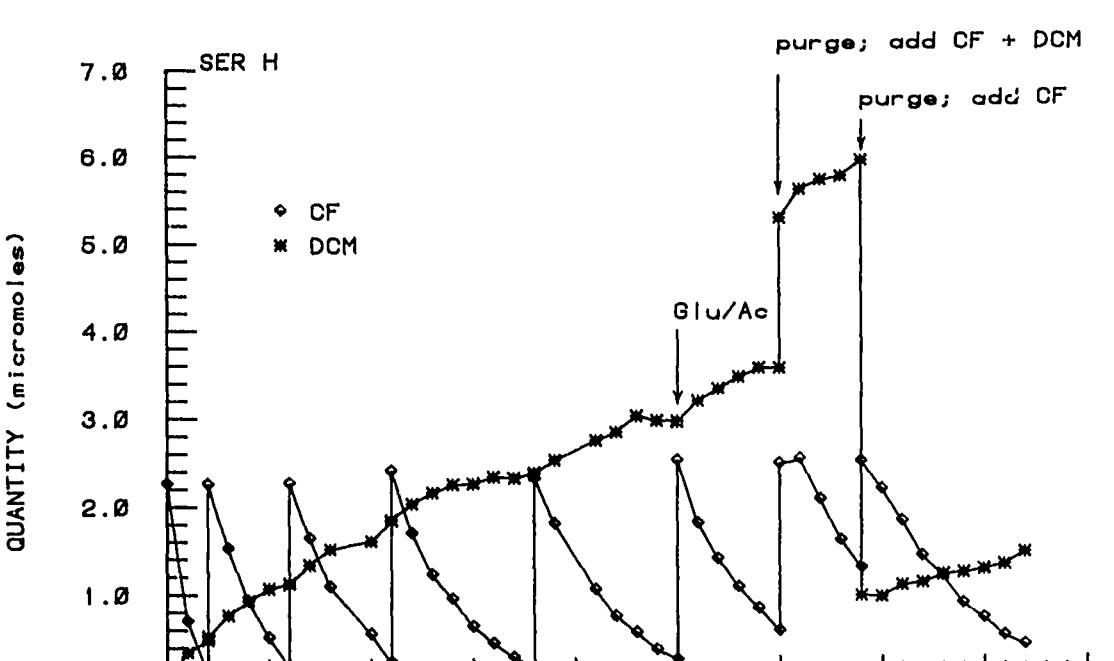

Figure 23. Repetitive-Addition Studies With Chloroform.

procedure was finally carried out: the bottle was purged, sealed, and respiked with CF at t = 34 days. The time required to effect the subsequent reduction of CF from 2.25 to 0.5 micromoles was about 5.5 days. Thus, no improvement in utilization rate resulted from the stripping of DCM from the bottle.

A plausible explanation for the decrease in utilization rates with successive additions of CF is that the system was limited either by the scarcity of some unknown growth factor (a vitamin, metal, or other cofactor), or by the accumulation of some inhibitory product. All that is suggested by these results is that the growth factor is neither glucose nor acetate (or any intermediates/products derived from them); and that the accumulated, inhibitory product is probably not DCM.

F. DISCUSSION

In this research, microbial utilization of CF under anaerobic conditions was demonstrated to proceed without acclimation. This suggests that CF is processed by a constituitive enzyme system. Since CF is not a "natural" substrate, its metabolism may result merely from its resemblance to some other, commonly encountered substrate or metabolite. An important question is whether organisms within the mixed, anaerobic culture were deriving energy or other growth-related benefit from utilization of CF. Unfortunately, the answer is unclear.

Results from studies employing repetitive CF additions suggest that growth may not have occurred on CF. Its utilization rate diminished over successive additions. However, alternative explanations (e.g., rate limitation resulting from scarcity of other growth factors or accumulation of inhibitory products) cannot be dismissed. Additionally, there is the observation that from 12 to 17 percent of the added ^{14}C was ultimately associated with the solids (Table 7); this may reflect the conversion of radiolabeled CF into cellular carbon. However, it could also reflect sorption.

1. Biochemical Pathways of Methanogenesis From C_1 Compounds

The microorganisms responsible for the observed degradation of CF in these studies have certainly not yet been identified. However, given that a methanogenic mixed culture was employed, it is logical to suspect methanogens. This group of bacteria is widely involved in metabolism of other C_1 compounds (e.g., formate, methanol, carbon dioxide). Before he indulges in further speculative discussion, the author will present some useful background information concerning the known metabolic pathways of methanogenic bacteria. These pathways will provide the context in which to consider some possible schemes by which methanogens might process CF and DCM.

Figure 24 is an attempt to summarize the current state of our knowledge regarding the metabolism of C_1 compounds by methanogenic bacteria. The pathways shown reflect the author's distillation of several recent reviews of this subject by other investigators (References 39, 40, 41, and 42). There is disagreement among the published reviews with respect to some pathways (e.g., those involving conversion of methanol to methane). However, there is general agreement on the basic scheme depicted in the figure. It should be additionally noted that, although several C_1 substrates are shown in this one figure, no known, single species is capable of metabolizing all of them.

Two unique, key carriers of C_1 groups have been identified in methanogens: CDR (carbon dioxide reduction factor, methanofuran); and FAF (formaldehyde activation factor, tetrahydromethanopterin). These two appear to be involved in the transfer of C_1 groups through the various oxidation states to and from (-II) to (+IV).

Methanogenesis from H_2 and CO_2 proceeds as follows. H_2 is oxidized, yielding two electrons which are transferred to an electron carrier (represented by [2H]), plus at least one proton (the other may accompany the two electrons to the carrier, as in the reaction, $H_2 + NAD^+ \Rightarrow NADH + H^+$). The hydrogenase involved is thought to be associated with the cellular membrane, with expulsion of the protons from the cytosol, serving to create a protonmotive driving force for phosphorylation of ADP via chemiosmotic coupling. Substrate-level ATP production is rare or absent. The [2H] reducing power from H_2 is used

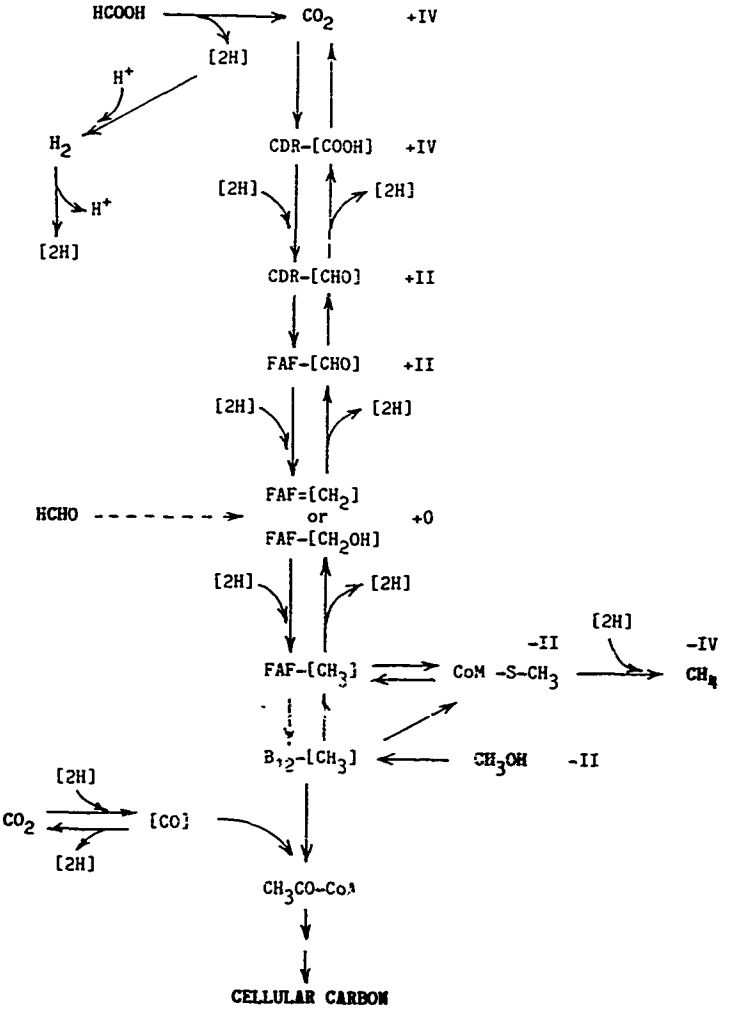

Figure 24. Biochemical Pathways of Methanogenesis From C_1 Compounds.

to bring about the conversion of CO_2 to CH_4, the last step of which involves the methanogenic coenzyme M (2-mercaptoethanesulfonic acid, abbreviated CoM-SH), possessing a terminal thiol group which can accept a methyl group from FAF-$[CH_3]$ via nucleophilic attack of the sulfur. The methyl group of CoM-S-CH_3 is reduced to CH_4 by the action of methyl-Co-M reductase and an electron carrier.

Methanogenesis using formate as electron donor proceeds in a manner very similar to that described above. Oxidation of formate to CO_2 provides reducing power; but, curiously, CH_4 results from subsequent reduction of CO_2, and not from reduction of formate. On the other hand, CH_4 is derived from the reduction of methanol, using electrons removed in the oxidation of methanol to CO_2. Figure 24 also shows the level at which formaldehyde enters the metabolic scheme. Methane has been shown to result from addition of formaldehyde in cell-free extracts, but no species is known to use this substrate. Formaldehyde freely reacts with FAF in a rapid equilibrium with FAF=$[CH_2]$.

Synthesis of cellular constituents from C_1 substrates occurs via acetyl-CoA, which is produced by combining a methyl group, transferred from B_{12}, with a carbon at the [CO] level, derived from CO_2.

2. Hypothetical Pathways of CF Metabolism in Methanogenic Systems

If methanogens were degrading CF, a plausible model of the pertinent metabolic pathways might be that depicted in Figure 25. Clearly any model presented in the absence of hard evidence must be regarded as speculative. The model of Figure 25 was developed by extension of the known, methanogenic pathways and is consistent with them, in terms of its use of transfer factors which serve as carriers of C_1 groups. The author is not brazen enough to tentatively identify these transfer factors by any symbol other than "X." The model is also consistent with the known chemistry of chlorinated C_1 compounds. Furthermore, many of the pathways are biochemically unidirectional — and are depicted as such —because they involve dechlorination.

CF is shown to enter metabolism at the level of X-$[CHCl_2]$ by elimination of HCl. An example of such a reaction might be the following nucleophilic substitution:

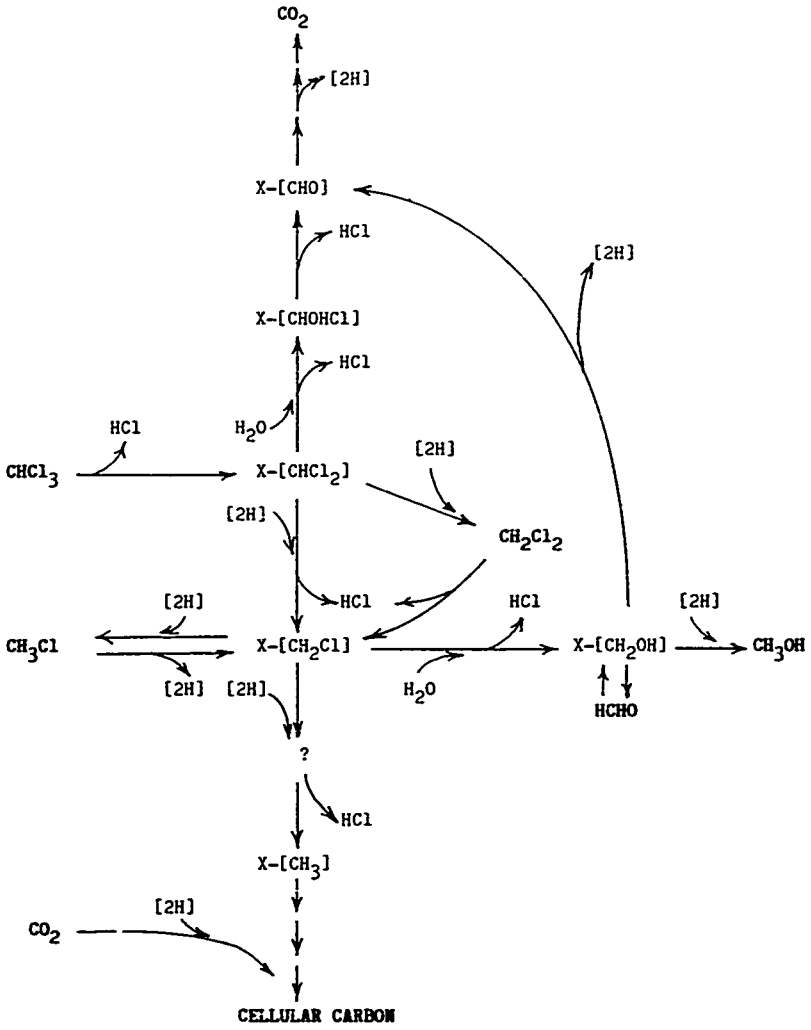

Figure 25. Hypothetical Pathways for Metabolism of Chloroform by Methanogenic Systems.

$$CoM\text{-}S\text{-}H + CHCl_3 \Rightarrow CoM\text{-}S\text{-}CHCl_2 + HCl \quad (8)$$

Chloroform is thought to inhibit methanogenesis by interfering with the production of CH_4 from CoM-S-CH_3 (the methyl-Co-M-reductase-mediated step) (Reference 40); reaction of CoM-S-H with CF could be one possible explanation.

Once CF has entered the metabolic system as X-[$CHCl_2$], further dehalogenation would likely occur in a manner analogous to the suspected pathway of DCM metabolism in aerobic bacteria, which proceeds via a halidohydrolase, resulting in monochloromethanol which spontaneously forms formaldehyde. In the case of CF metabolism, the model speculates a similar dehydrohalogenation/hydration resulting in X-[CHO]. From this point, oxidation to CO_2 may follow established pathways (see Figure 24).

At the level of X-[$CHCl_2$], reductive dechlorination may result in liberation of DCM, or the carrier-bound form, X-[CH_2Cl]. Reductive dechlorination of the latter could result in formation of free CM or the carrier-bound form, X-[CH_3]. However, a question mark has been inserted at this point, because experimental evidence tends not to support the formation of carrier-bound, CF-derived carbon in the (-II) state. If X-[CH_3] were formed, its only conceivable use would involve either methane production or anabolic production of cellular consituents. Unless methanogenesis is otherwise inhibited (which may be the case with CF), anabolism from CF carbon would necessarily pass through intermediates (FAF-[CH_3] and/or B_{12}-[CH_3]) common to both anabolic and methanogenic pathways, which should result in formation of significant amounts of methane directly from CF carbon. Results from this research do not conflict with the hypothesized anabolic use of carbon from CF; but direct methanogenesis from CF carbon has not been observed in either this study, or that of Bouwer and McCarty (Reference 26). It appears more likely that anabolism and methanogenesis both proceed via reduction of CO_2. CF metabolism may be restricted to its oxidation, providing the reducing power and protonmotive force required for methanogenesis and anabolism from CO_2. Thus, the oxidative pathways from X-[CH_2OH] to CO_2 may be irreversible (or at least physically and/or biochemically

uncoupled from the similar, FAF- and CDR-mediated steps depicted in Figure 24).

Figure 25 also shows a possible means for the metabolism of DCM, using a reductive dechlorination/hydration sequence similar to that reported by Stucki et al. (Reference 25) from their studies with a DCM-degrading Pseudomonas sp. Once dechlorinated to X-[CH_2OH] (or X=[CH_2]), further oxidation to CO_2 would be possible as in conventional pathways (see Figure 24); alternatively, formaldehyde might be liberated, as was observed by Stucki et al. in their studies. In cell-free experiments, FAF=[CH_2] has been shown to rapidly equilibrate in a reversible reaction with FAF + HCHO (Reference 41).

It is further speculated that the accumulations of DCM and CM observed in these studies may have resulted from the inhibition of methanogenesis by CF. That such inhibition occurred is evident from gas-production data. With CH_4 production inhibited, some other electron-accepting reaction would have to serve as a sink for the electrons removed from CF in its oxidation to CO_2. Production of DCM, CM, HCHO and/or CH_3OH from CF would serve this purpose. While accumulations of DCM and CM were observed, productions of HCHO and CH_3OH are merely conjectured. However, such productions would be consistent with the significant, soluble ^{14}C levels observed in sample residues following extensive N_2 stripping at low pH. The above hypothesis that DCM and CM production resulted from inhibition of methanogenesis may also explain one significant difference between these results and those of Bouwer and McCarty (Reference 26), who observed virtually complete oxidation of CF to CO_2, with no DCM reported. Bouwer and McCarty used a CF concentration of approximately 30 micrograms per liter, versus the 1.7 to 17 mg/L (considering partitioning of CF to bottle headspaces) employed here. Inhibition is not likely to have been significant in their studies.

3. Energetics

Free energies of some pertinent half reactions are contained in Table 8. Coupling them in different ways allows estimation of the thermodynamic energies available from prospective conversions of CF:

TABLE 8. PERTINENT HALF REACTIONS[a]

	$\Delta G^{o}(W)$[b] (kcal)
Hydrogen	
$H^+ + e^- = 1/2\ H_2$	9.549
Methane	
$1/8\ CO_2 + H^+ + e^- = 1/8\ CH_4 + 1/4\ H_2O$	5.645
Chloroform	
$1/2\ CO_2 + 3/2\ Cl^- + 5/2\ H^+ + e^- = 1/2\ CHCl_3 + H_2O$	53.58
Dichloromethane	
$1/4\ CO_2 + 1/2\ Cl^- + 3/2\ H^+ + e^- = 1/4\ CH_2Cl_2 + 1/2\ H_2O$	21.23
Chloromethane	
$1/6\ CO_2 + 1/6\ Cl^- + 7/6\ H^+ + e^- = 1/6\ CH_3Cl + 1/3\ H_2O$	10.89

[a] Half-reaction free energies were calculated from free energy of formation data compiled by Stumm and Morgan (Reference 43), except that such data were unavailable for aqueous CF, DCM, and CM. Free energies of formation for these three were calculated using standard free energies of formation for the pure, gaseous forms (Reference 44), converting the values to aqueous conditions at unit activity by employing regression values of Henry's constants (Table A-3) in the following relation:

$$\Delta G^{o}_{aq} = \Delta G^{o}_{g} + RT \ln H$$

where ΔG^{o}_{aq} = standard free energy of formation of the aqueous species;
ΔG^{o}_{g} = standard free energy of formation of the pure gaseous species;
H = Henry's constant (liter-atm/mol);
R = gas constant [1.987 cal/(mol·°K)];
T = 298 °K.

[b] 25°C, 1 atm, with all species in their standard states at unit activity, except $\{H^+\} = 10^{-7}$, assuming pH 7 conditions.

$$CHCl_3 + 3/2\ H_2O = 3/4\ CO_2 + 1/4\ CH_4 + 3\ Cl^- + 3\ H^+ \quad (9)$$

$$\Delta G^o(W) = -95.87 \text{ kcal.}$$

$$CHCl_3 + 4/3\ H_2O = 2/3\ CO_2 + 1/3\ CH_3Cl + 8/3\ Cl^- + 8/3\ H^+ \quad (10)$$

$$\Delta G^o(W) = -85.38 \text{ kcal.}$$

$$CHCl_3 + H_2O = 1/2\ CO_2 + 1/2\ CH_2Cl_2 + 2\ Cl^- + 2\ H^+ \quad (11)$$

$$\Delta G^o(W) = -64.70 \text{ kcal.}$$

Solely from considerations of thermodynamics, one would expect accumulation of CM, rather than DCM, if methanogenesis were inhibited by CF, requiring a shift to another electron-accepting process. However, considerations of kinetics might dictate otherwise. The data of Figure 12 do seem to indicate the eventual conversion of DCM to CM.

It is possible that CF oxidation is not mediated by a methanogen at all, but by a H_2-evolving organism. Perhaps a second organism — the methanogen — then uses the H_2 to reduce CO_2 to CH_4. But in the presence of inhibitory CF, the methanogen requires a different electron-accepting reaction. If this were the case, then the energetics should be examined from a different perspective: which of the two conversions (CF to CM, or CF to DCM) yields the greater potential energy as an electron-consuming process:

$$1/2\ CHCl_3 + 1/2\ H^+ + e^- = 1/2\ CH_2Cl_2 + 1/2\ Cl^- \quad (12)$$

$$\Delta G^o(W) = -11.12 \text{ kcal.}$$

$$1/4\ CHCl_3 + 1/2\ H^+ + e^- = 1/4\ CH_3Cl + 1/2\ Cl^- \quad (13)$$

$$\Delta G^o(W) = -10.46 \text{ kcal.}$$

From this consideration, DCM would appear to be preferred as the product of CF reduction.

SECTION VII

ANAEROBIC DEGRADATION OF DICHLOROMETHANE

A. EXTENDED INCUBATION

A 9.0-micromole dose of dichloromethane (DCM) was injected to seeded-sample, ASC, and WC systems, with subsequent incubation for 93 days. The added amount corresponds to a nominal, aqueous concentration of about 7.6 mg/L. When partitioning to the gas phase is considered, the initial solution concentration from such a dose would have been approximately 7.1 mg/L. WC and seeded-sample systems were prepared in duplicate, but sampling was generally alternated between members of a duplicate pair on successive days. Results are shown in Figure 26. Neither sorption nor leakage of DCM was apparent in this phase of study, as is evident from examination of WC and ASC data.

After a lag of from 6 to 9 days, DCM was rapidly degraded in the seeded samples. After resting in the absence of DCM for one day, the seeded systems were again spiked with DCM. It was expected that the originally-observed lag might now be absent — or at least lessened — by previous exposure of the microorganisms to DCM. However, the data indicate a second lag of approximately 9 days, followed by the rapid depletion of DCM. A third addition of DCM, after at least an 8-day absence of the compound, resulted in no further degradation. Later studies (described in Section VII-B) demonstrate that it is quite possible to maintain the culture in an acclimated condition, but that the acquired ability of the microorganisms to degrade DCM is rapidly lost in the absence of the compound.

A relatively small amount (0.18 micromoles) of CM was observed during acclimation periods. No other, possible products were detected. MeSH accumulated, but previous studies with CF (see Section VI-B) — as well as radiotracer studies discussed in this chapter — have demonstrated that MeSH accumulation is likely the result of inhibition of methanogenesis. Note, for example, that MeSH levels decreased during every interval when DCM was absent, and increased during periods of high DCM levels. The increases in MeSH were obviously not at the expense of

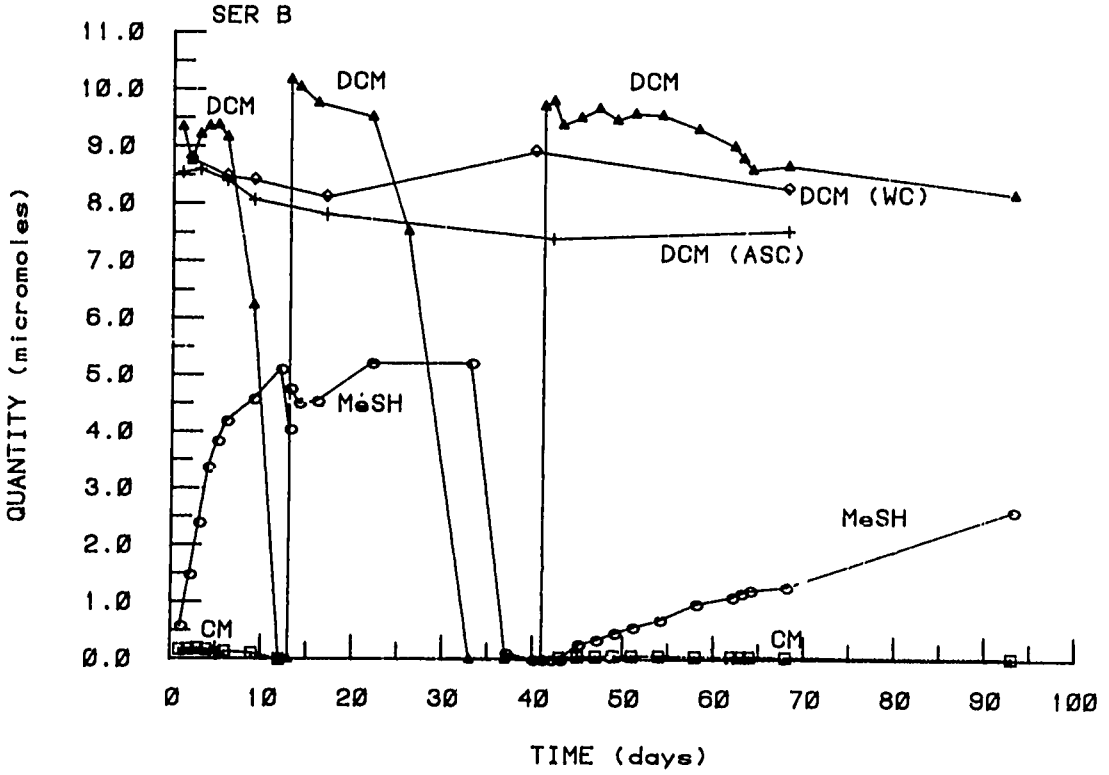

Figure 26. Extended Anaerobic Incubation of Dichloromethane (DCM).

DCM, which often sustained a relatively constant level over such periods (e.g., note data of the first 6 days of incubation).

B. REPETITIVE ADDITIONS OF DICHLOROMETHANE

The pattern of acclimation to DCM was investigated further, using three different, added levels: 2.9, 9.6, and 29 micromoles. Bottles at each level were prepared in triplicate, with one bottle used for measurement of gas production, while the other two were employed in the repetitive-addition studies.

Gas production data are shown in Figure 27. The bottles which served as the basis for these data were dosed initially with DCM, and not subjected to respiking. Inhibition of gas production was significant at the two highest DCM levels, though not as severe as was noted in previous studies with CF.

Typical results from acclimation/repetitive-addition experiments are shown in Figure 28. For purposes of clarity, MeSH data have been omitted. After a lag of approximately 15 days, DCM was rapidly degraded over a period of 3 days. Just before its complete disappearance, the system was respiked (t = 18 days), and subsequent removal of DCM proceeded without a lag, with the added 30 micromoles consumed in less than 3 days. The DCM level was allowed to remain below detection limits for at least 4 hours before adding additional DCM (t = 21 days). A lag of about 4 days resulted, followed by the rapid depletion of DCM.

Similar results were obtained with repeated additions of DCM over the remainder of the observation period. Whenever the bottle was respiked prior to the complete disappearance of DCM, subsequent degradation continued at a high rate; whenever the microbial system was allowed to completely deplete the DCM, a lag period resulted. An acclimation period of 7 days was required following a 6-hour interval spent in the absence of DCM (at approximately t = 33 days).

No correlation was evident between the time required for initial acclimation and the level of added DCM; between 15 and 20 days were initially required by all bottles at all DCM levels. In fact, the initial lag periods of duplicate bottles were observed to be at both extremes of the 15-to-20-day range cited above.

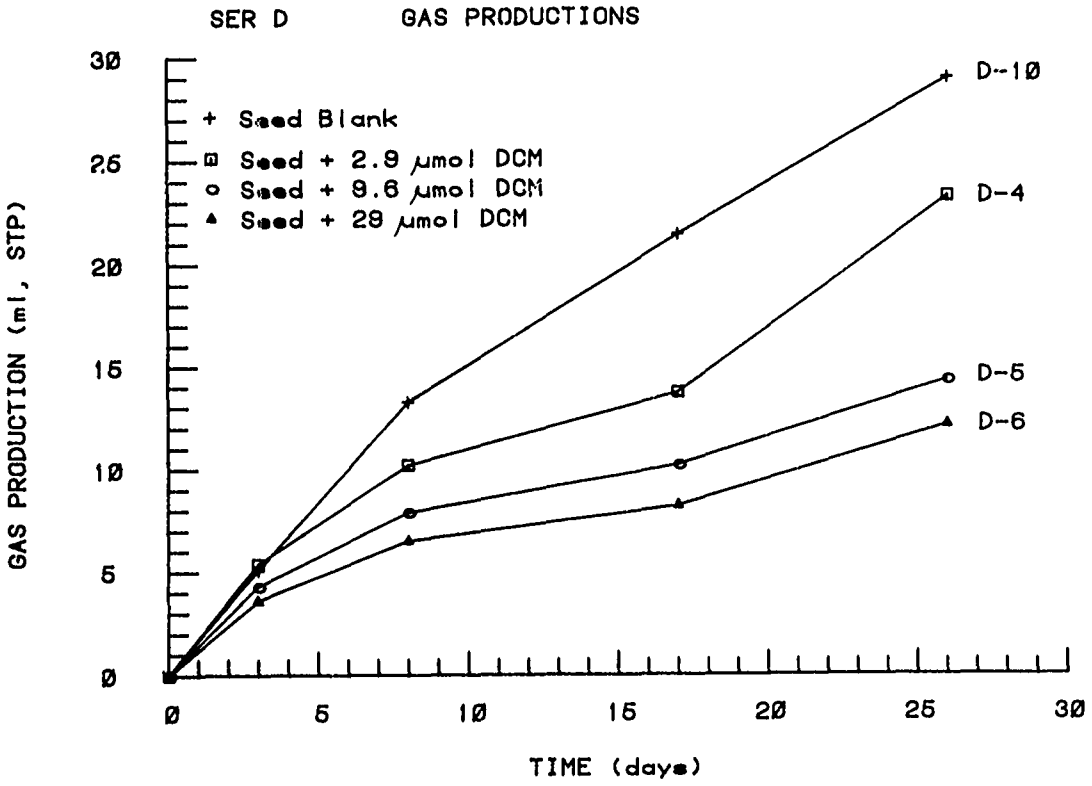

Figure 27. Effect of Dichloromethane Level on Cumulative Gas Production.

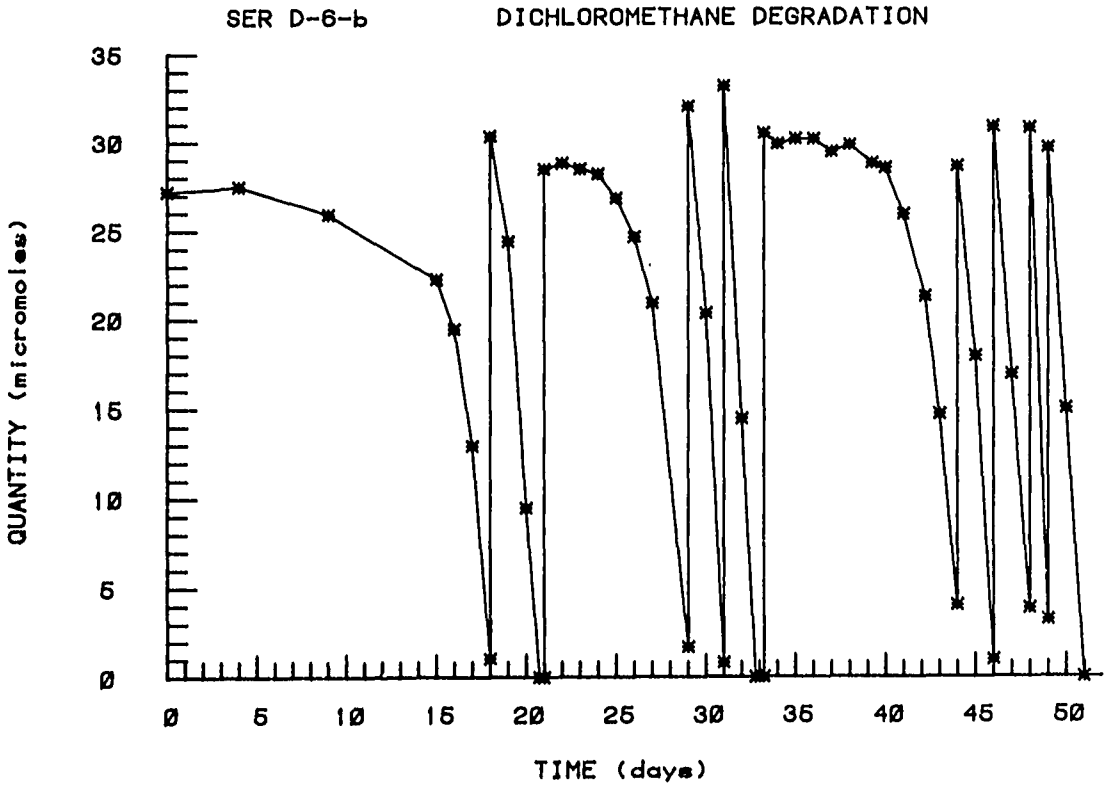

Figure 28. Repetitive-Addition Studies With Dichloromethane.

C. INHIBITION OF DCM DEGRADATION BY ADDITION OF CF

In previous studies with CF (Section VI), its degradation resulted in the accumulation of DCM for extended periods. Results from these present studies with DCM, however, demonstrate its rapid degradation, following an initial acclimation period. Further experiments were undertaken to resolve this apparent discrepancy.

Figure 29 shows data from another of the systems employed in the repetitive-addition experiments described in Section VII-B. The trend is the same as that observed above: sustained acclimation in the presence of DCM, with rapid loss of acclimation during periods of its absence.

At t = 43 days -- one of those times when DCM had been depleted to a low, but still significant level -- additions of DCM and CF were made. The added CF quantity was about 2 micromoles, a level sufficient to produce significant inhibition of methanogenesis (Figure 18). The result, as evident in Figure 29, is that DCM degradation ceased, though the microorganisms had not been deprived of DCM. Inhibition of DCM degradation persisted long after CF itself had disappeared from the system until approximately t = 60 days (not shown in Figure 29) had elapsed. Utilization of DCM did not resume.

CF appears to inhibit utilization of DCM in a manner that is only slowly reversible. In previous studies with CF-fed systems, DCM was also seen to accumulate for extended periods -- long after CF had apparently disappeared. The implication is that some CF may bind almost irreversibly to important metabolic factors, persisting at low levels long after CF has been removed from bulk solution. Another possibility is that CF causes acute toxicity (i.e., death). The sole remedy might be introduction of fresh, uninhibited microorganisms after depletion of CF from bulk solution.

Figure 30 illustrates the effect of reinoculation. A CF-fed system exhibited the usual depletion of CF, with production of DCM, which persisted for some 25 days at relatively constant level. At t = 31.25 days, 5 mL of mixed liquor was injected from bottle D-5-a, a system acclimated to degradation of DCM. Within 2 days, degradation

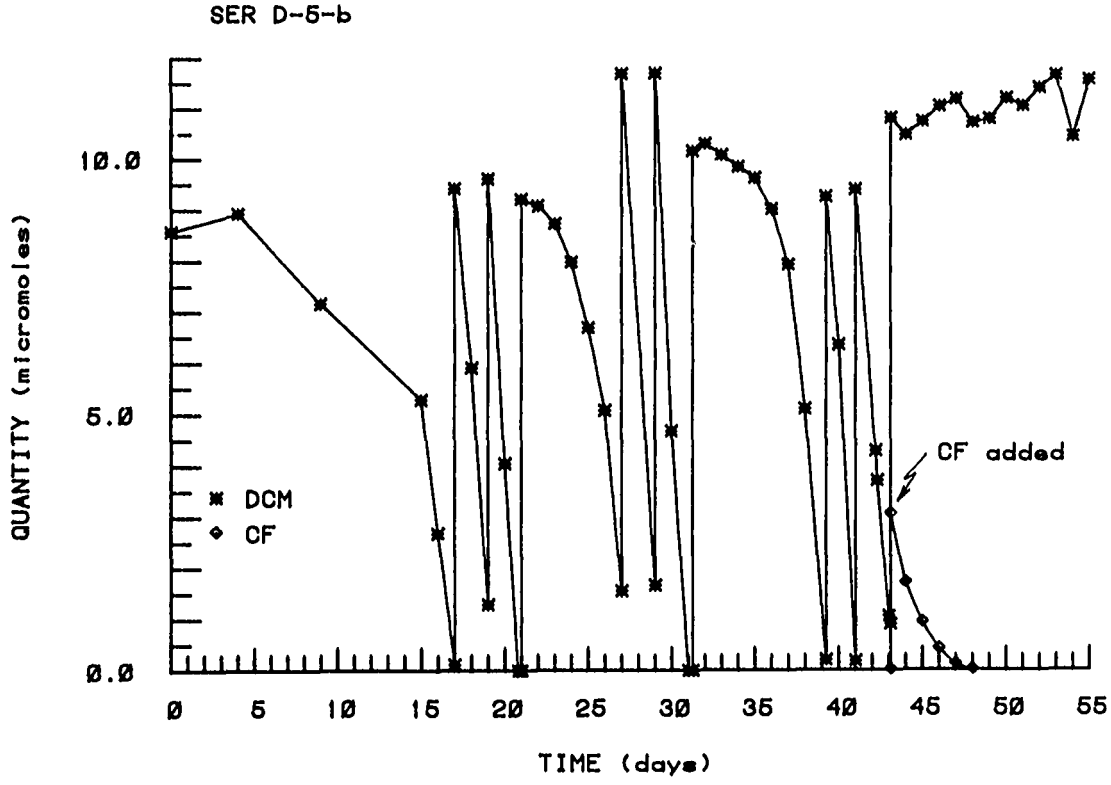

Figure 29. Repetitive-Addition Studies With Dichloromethane (Chloroform Added at t=43 Days).

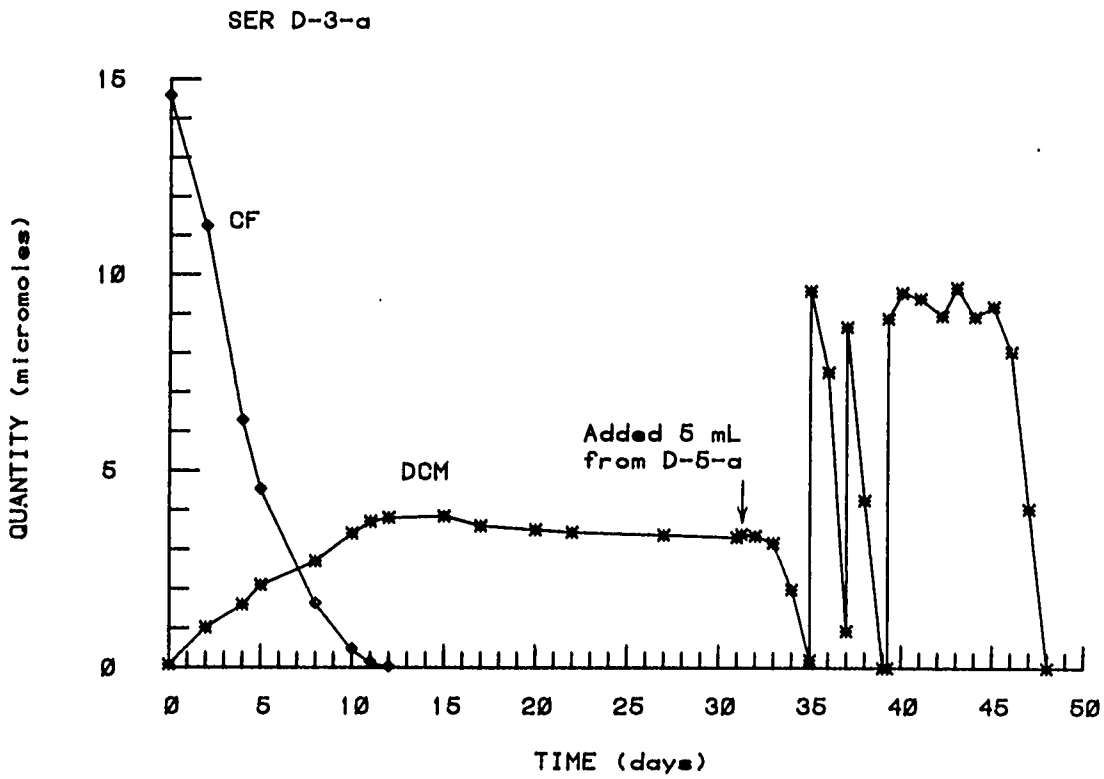

Figure 30. DCM Degradation Following Re-inoculation of a CF-Inhibited System.

of the persistent DCM commenced at a relatively rapid rate. DCM was almost totally depleted within another two days. The bottle was spiked with DCM at t = 35 days -- just prior to depletion of DCM -- and proceeded to exhibit the previously observed response to repetitive additions: sustained acclimation and utilization of DCM at a high rate, so long as the microorganisms were in the presence of even modest amounts of the compound; and an extended lag period resulting from a mere 4-hour period in the absence of DCM.

D. STUDIES WITH RADIOTRACERS

Unfortunately, radiolabeled DCM was not available. The only means of employing radiotracers in the study of DCM metabolism were indirect ones: since degradation of CF results in significant production of DCM, the addition of ^{14}C-CF to systems might possibly yield information concerning the metabolic fate of DCM -- but only if the microorganisms could be induced to degrade the ^{14}C-DCM formed from ^{14}C-CF. Two approaches were used.

In one instance, ^{14}C-CF was added to a DCM-acclimated system (bottle D-6-a) while DCM was being actively degraded (Figures 31 and 32). Within 3 days, when DCM was no longer detected and most of the ^{14}C-CF had also been degraded, ^{14}C-distribution analyses were conducted.

One possible criticism is that there is no hard evidence that any of the added CF was ever converted to DCM. After all, since the bottle was spiked with only the ^{14}C-CF stock solution, the resulting peak level of CF was low (about 0.2 micromoles). While the CF level was purposely kept low so as to prevent inhibition of DCM degradation, it is possible that no ^{14}C-DCM was ever produced. (Recall that in Section VI-F-2, it was suggested that DCM may result from degradation of CF only because of CF's inhibition of methanogenesis.) Therefore, what may have been investigated in this case is merely the metabolic fate of CF in the absence of inhibition of methanogenesis.

Therefore, a second approach was additionally employed. DCM, ^{14}C-CF, and stable CF were simultaneously added to a DCM-acclimated system (bottle D-4-b). The added level of total CF was approximately 1.5 micromoles -- a level which previous study had demonstrated would

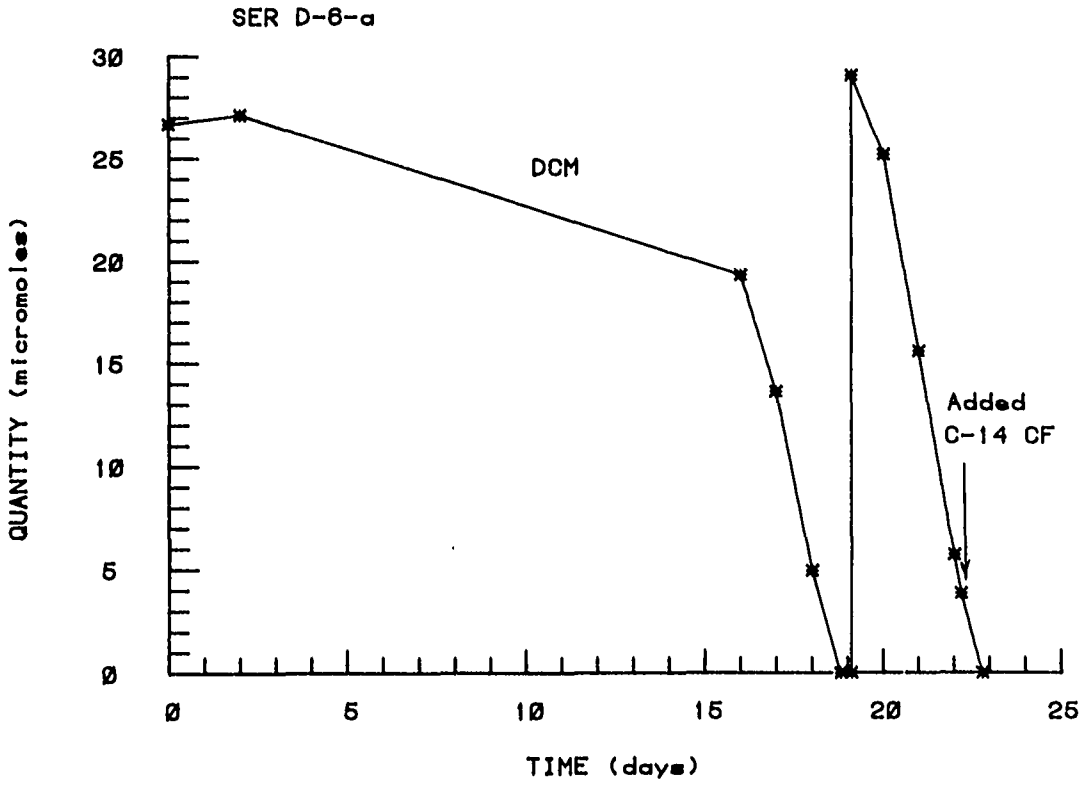

Figure 31. ^{14}C-CF Addition to Bottle D-6-a.

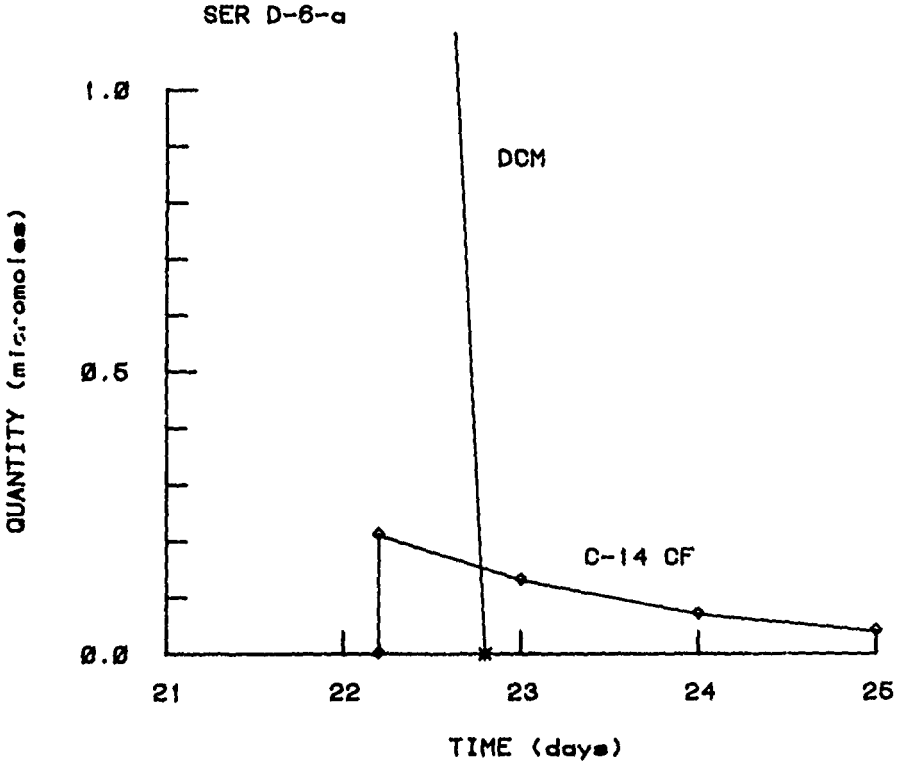

Figure 32. ^{14}C-CF Addition to Bottle D-6-a (Detail).

both inhibit methanogenesis (Figure 18) and cause production of DCM from CF (Figure 19). In this instance, the added CF also inhibited DCM utilization (Figure 33). To relieve this, the bottle was inoculated with 5 mL of mixed liquor from D-4-a (a DCM-acclimated system). Over the following 5 days, DCM was completely degraded.

In retrospect — given that the CF was going to cause inhibition of DCM utilization, necessitating reinoculation -- it would have been preferable to have performed these ^{14}C studies by adding stable and radiolabeled CF in the absence of pre-existing levels of DCM. The subsequent appearance of DCM would be positive proof of its formation from CF. Inoculation with a DCM-acclimated culture could then result in degradation of the DCM, followed by the determination of ^{14}C species.

Results from the ^{14}C studies are summarized in Table 9. On the basis of the preceding discussion, there are fundamental reasons to believe that the second system may have provided data which more accurately reflect sequential conversion of CF to DCM to (apparently) CO_2. However, results from the two methods of ^{14}C-CF addition do not significantly differ. Both systems exhibited negligible productions of CH_4. DCM is apparently substantially oxidized to CO_2.

Earlier results from ^{14}C-CF-fed systems which accumulated DCM (Table 7) can be used in conjunction with results in Table 9 to estimate the metabolic fate of DCM in the present studies. Results of the calculation depend upon what percentage conversion of CF to DCM is assumed to have occurred in D-6-a and D-4-b. Data in Table 7 suggest that this percentage is influenced by the CF level and/or time of incubation after adding the CF. The D-1-b system of Table 7 is comparable to the D-4-b system of Table 9; each was dosed with approximately the same level of CF (1.5 vs. 1.6 micromoles), which was totally consumed within about three days in both cases. Therefore, it is assumed that both systems exhibited approximately the same percentage conversion of CF to DCM (i.e., 34.1 percent). The D-1-b system converted 43.5 percent of its CF to CO_2, versus 72.8 percent for the D-4-b system. This implies that approximately 86 percent (i.e., 72.8 units minus 43.5 units, divided by 34.1 units) of the eventually metabolized DCM was converted to CO_2.

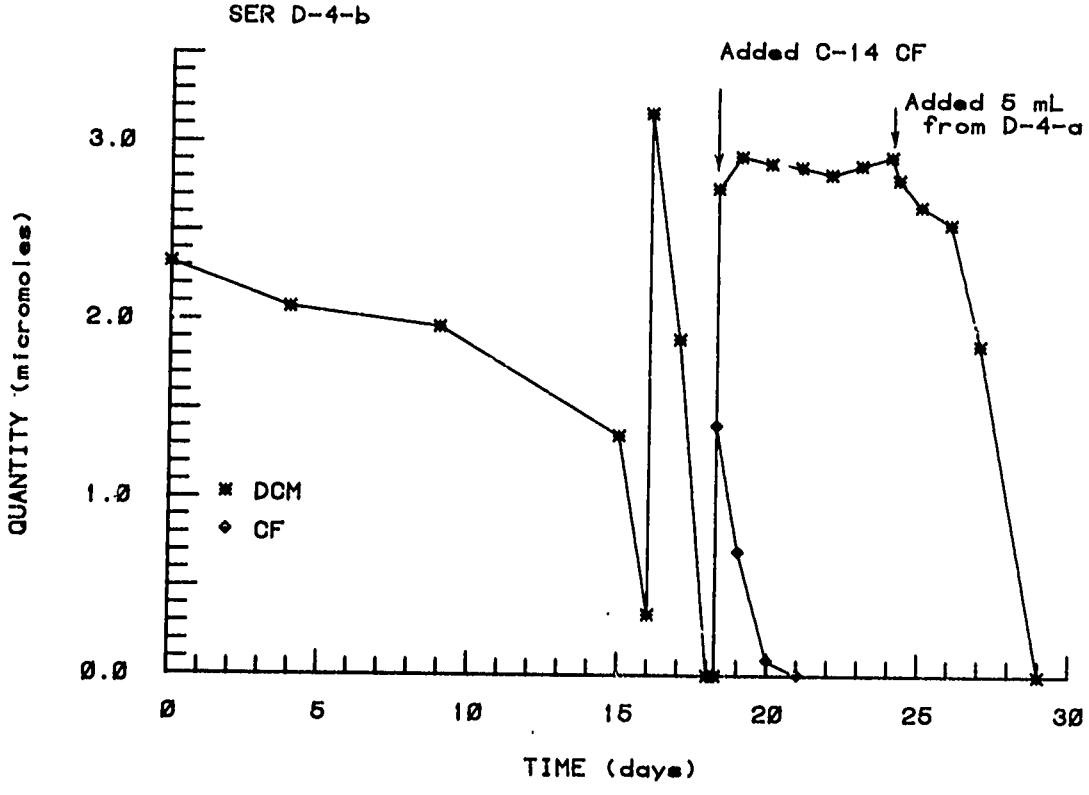

Figure 33. ^{14}C-CF Addition to Bottle D-4-b.

TABLE 9. DISTRIBUTION OF ^{14}C IN DCM-DEGRADING SYSTEMS FED LABELED CF

Bottle	Fraction ID	dpm	% of added dpm[a]
D-6-a	CF	373,000	14.8
	CM	0	0
	DCM	20,000	0.8
	MeSH	0	0
	CH_4	2,640	0.1
	CO_2	1,760,000	69.6
	Nonstrippable Residue	402,000[b]	15.9
	Total	2,557,640	101.2
D-4-b	CF	0	0
	CM	0	0
	DCM	0	0
	MeSH	0	0
	CH_4	3,560	0.1
	CO_2	1,840,000	72.8
	Nonstrippable Residue	539,000[c]	21.3
	Total	2,382,560	94.2

[a] 2.528×10^6 dpm ^{14}C-CF added.

[b] Of this total, 47 percent was associated with the soluble phase.

[c] Of this total, 50 percent was associated with the soluble phase.

A similar calculation can be performed by comparing results from D-6-a and D-1-b. The added levels of CF were significantly different in the two instances (0.2 vs. 1.6 micromoles); but the incubation periods prior to analysis were approximately the same. In this instance, the calculation must take account of the unconsumed CF in D-6-a: Of 100 units of CF added, only 85.2 units were consumed, resulting in 69.6 units of CO_2 and 0.8 units of DCM. This implies that approximately 92 percent (i.e., [69.6 - 43.5]/[0.852 x 34.1 - 0.8]) of the eventually metabolized DCM was converted to CO_2.

E. ENRICHMENTS

To better define the organism(s) responsible for the observed degradation of DCM, and to study their requirements, an enriched culture of such organisms is desirable. Dilution of uninvolved organisms and extraneous organic matter present in the sewage-sludge inoculum, while increasing the proportion of DCM-utilizing bacteria, should allow types of investigation which would otherwise be impossible (e.g., studies of biochemical pathways). Success in developing an enrichment would also provide strong evidence that DCM serves as a growth-linked substrate.

1. Procedures

The basal medium used is described in Table 10. Its composition was designed under the assumption that the organisms whose enrichment is sought are methanogenic bacteria. The formulation is a heavily modified version of one used by Zeikus (Reference 45). The major differences are that Zeikus' medium employs a phosphate-based buffer system, whereas the one used here is buffered by the bicarbonate system. Consequently, the phosphate concentration has been decreased by 90 percent. Also, nickel has been added to the trace-metal solution, reflecting the recognized need which methanogens have for this element; and the quantity of ferrous iron has been greatly increased.

Medium preparation consisted of the following steps. The ammonium chloride, potassium phosphates, magnesium chloride, trace-metal solution, and resazurin were added to distilled water in a flask, along

TABLE 10. BASAL MEDIUM FOR DCM ENRICHMENT

Component	Quantity
NH_4Cl	0.20 g
$K_2HPO_4 \cdot 3H_2O$	0.10 g
KH_2PO_4	0.055 g
$MgCl_2 \cdot 6H_2O$	0.20 g
TMS[a]	10 mL
$Na_2S \cdot 9H_2O$	0.50 g
$FeCl_2 \cdot xH_2O$	0.10 g
$NaHCO_3$	5.0 g
Yeast Extract	0.050 g
Resazurin	1 mg
Distilled H_2O	1000 mL

[a] Trace Metal Solution: 0.1 g/L $MnCl_2 \cdot 4H_2O$; 0.17 g/L $CoCl_2 \cdot 6H_2O$; 0.10 g/L $ZnCl_2$; 0.20 g/L $CaCl_2$; 0.019 g/L H_3BO_4; 4.5 g/L nitrilotriacetic acid (NTA); 0.05 g/L $NiCl_2 \cdot 6H_2O$; and 0.020 g/L $Na_2MoO_4 \cdot 2H_2O$. Adjusted to pH 7 with NaOH.

with a Teflon™-coated, magnetic-stirrer bar. The solution was brought to a boil while continuously stirred. A 100 mL/min flow of oxygen-free N_2 was passed over the surface of the solution via a cannula system. After 10 minutes of boiling, the flask was allowed to cool to near ambient temperature, while the N_2 purge continued. The sodium sulfide, ferrous chloride, and sodium bicarbonate were then added, and the composition of the purge gas was immediately switched to a 30 percent CO_2/70 percent N_2 mixture. The yeast extract was added, and then pH was checked (it was 7.5). The basal medium was now ready for inoculation and/or anaerobic transfer to 158.8 mL serum bottles.

2. Results to Date

The enrichment process was presumably begun with the D Series of bottles, where DCM was repeatedly added to a 100-percent digested-sewage-sludge inoculum. Those systems might be regarded as "first-generation enrichments" (assuming that growth occurred, and that enrichment is possible). A second-generation of enrichment was attempted using a rather large volume of inoculum from one of the earlier systems: 10 percent (by volume) of mixed liquor from a DCM-acclimated system (bottle D-5-a) was employed. At the time, this inoculum was actively degrading DCM. The inoculum was added directly to the flask of basal medium, which was subsequently transferred to serum bottles using the anaerobic transfer technique previously described (Section III-D-2). In this phase of study, a different seal (other than the usually used Teflon™-lined, rubber septum) was evaluated: a 13mm by 20 mm, slotted, gray, butyl-rubber stopper (Wheaton # 224154), held in place with an aluminum crimp cap. This new seal was evaluated because the Teflon™-rubber septa do not maintain their flexibility through autoclaving (a concern in later isolation studies). Furthermore, the slotted shape of the butyl-rubber stoppers is custom-made for the insertion and withdrawal of a gas cannula.

After sealing, each bottle was injected with 19 micromoles DCM from an oxygen-free, DCM-saturated stock solution. Results from this second generation of enrichment are shown in Figures 34 and 35 for two of the eight inoculated bottles which were prepared. The continuously

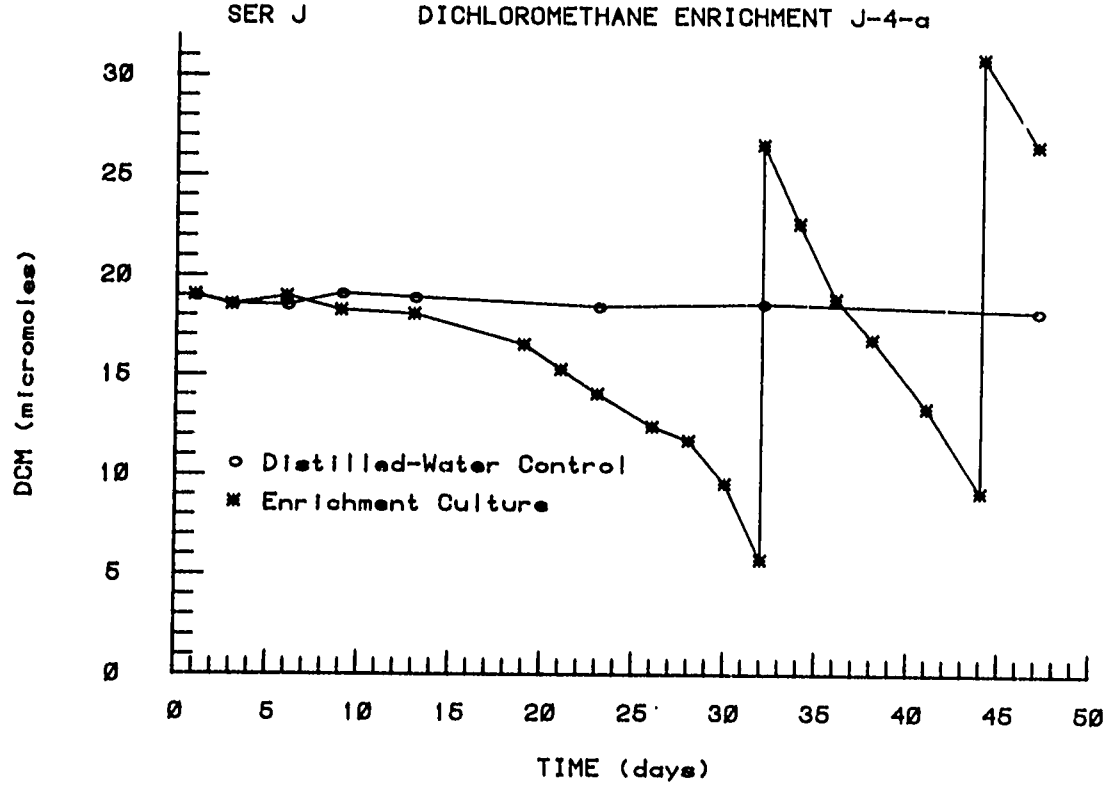

Figure 34. Series J-4-a DCM Enrichment.

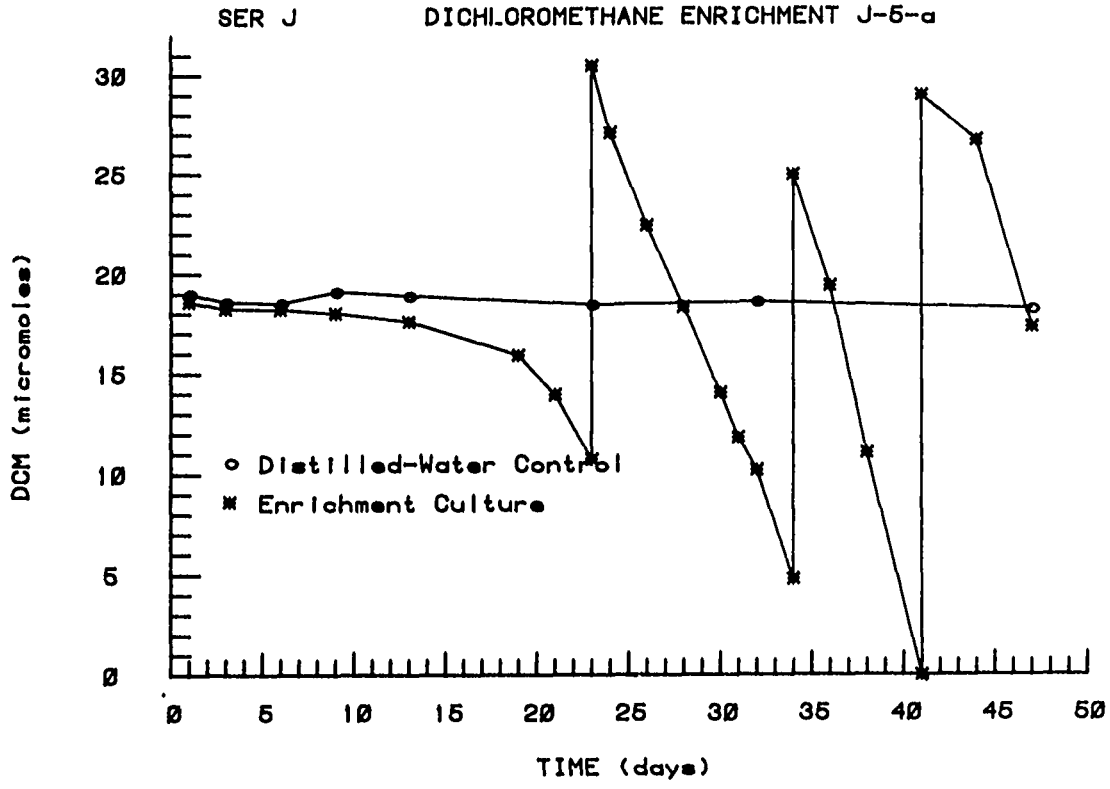

Figure 35. Series J-5-a DCM Enrichment.

increasing degradation rates evident in both Figures over the first cycle of degradation suggests that use of the term, "lag phase," may be inappropriate here. The initial pattern of utilization more resembles the manifestation of an exponential rate of increase in microorganism population, rather than a true "lag."

To date, the rates of DCM degradation have not approached those observed earlier with a 100 percent sewage-sludge inoculum. While the steadily increasing rates over the first degradation period are suggestive of exponential population increases, the relatively low ceiling to the degradation rate implies that some unknown factor may be limiting. Therefore, at t = 30 days, 50 mg/L of yeast extract was added to the J-5-a system. No dramatic effect was observed. Of course, all this proves is that the factor is not significantly present in yeast extract.

The butyl-rubber stopper has proven to be adequate for use with DCM; no significant loss of DCM occurred from a distilled-water control bottle over nearly 50 days of incubation (Figure 34 or 35). As with all incubated bottles in this research, storage was always effected with the bottle resting on its side, the seal in contact with the solution phase within the bottle.

Microscopic examination has thus far not been productive. No single morphological type appears dominant. This is not suprising; it is far too early to expect the appearance of a dominant population. Despite the repeated cycles of DCM addition prior to use of the inoculum in these enrichment studies, and despite the further additions of DCM, it must be remembered that the systems were fed DCM in tenths-of-millimolar doses; it should not come as a suprise if the population in these second-generation enrichments remains significantly influenced by its sewage-sludge origins.

Enrichment attempts continue. At the time of this writing, a third generation has been recently inoculated with 0.5 percent (by volume) mixed liquor from the J-5-a enrichment. A DCM level of 30 micromoles is being employed.

F. RELATIONSHIP BETWEEN DCM UTILIZATION AND METHANOGENESIS

The second-generation enrichments provided a relatively defined environment for studies not otherwise possible. One of these concerned the link between utilization of DCM and methanogenesis. Results from radiotracer studies discussed in Section VII-D indicated that DCM is not directly converted to methane. However, their indirect link had not been explored.

When the J Series of DCM enrichments was prepared (see previous subsection for details), any methane which might otherwise have been contributed by the mixed liquor of the D-5-a inoculum would most certainly have been stripped away. Thus, any CH_4 subsequently observed in the enrichments was formed by them; it might therefore be possible to observe a correlation between cumulative methane production and cumulative DCM utilization.

The possible production of methane from non-DCM sources contr'buted by the inoculum was a potential concern during data interpretation. Normally, an inoculated blank would be incubated in the absence of DCM, with its cumulative CH_4 values available to subtract from CH_4 values of samples. However, it is unlikely that the rate of methanogenesis from these extraneous sources would be the same in the presence of a potentially inhibiting level of DCM as it would be in the absence of DCM. No simple blank correction is possible. The logical approach is to assume that inoculum contributions would be the same in all DCM-containing sample bottles on a given day, presenting a constant-value error to observed, cumulative methane registered on that day.

On a single day, headspaces of all eight Series-J enrichments were carefully analyzed for CH_4 concentration via GC with TCD. Figure 36 presents the observed relationship between cumulative CH_4 production and cumulative DCM utilization. While the data are somewhat scattered, due to the imprecision associated with measurement of such trace levels of methane, the trend is clear: methanogenesis is linked to disappearance of DCM. The positive intercept of the regression line can be taken as an estimate of contribution made by organics in the inoculum to cumulative CH_4 production.

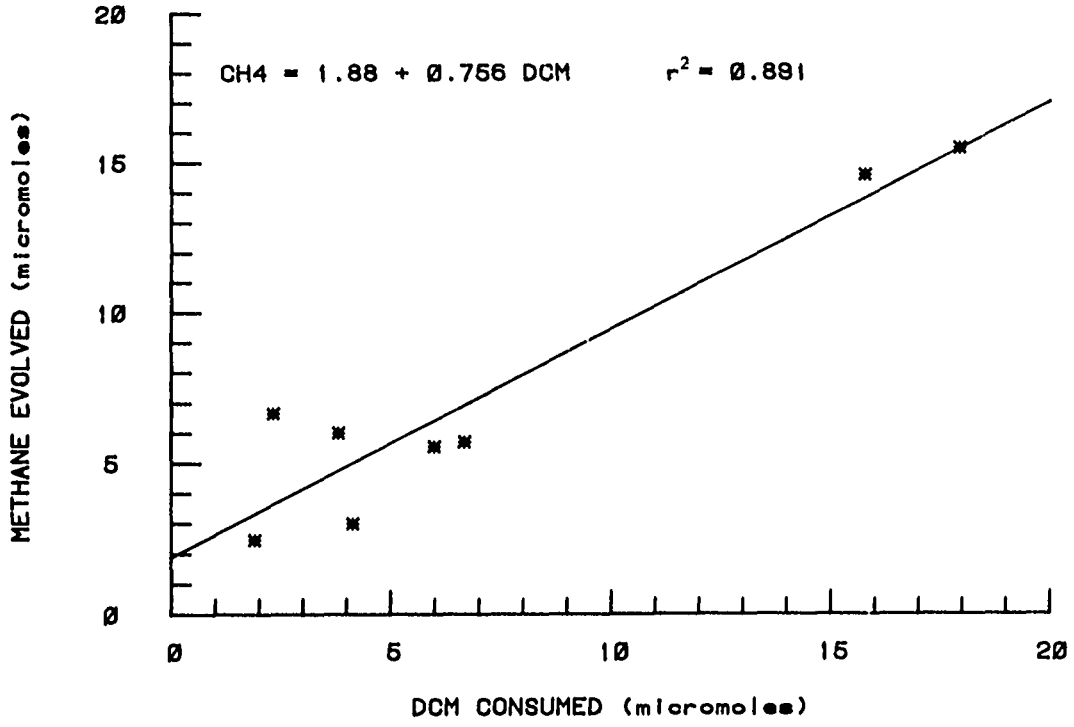

Figure 36. Relationship Between DCM Utilization and Methanogenesis.

G. DISCUSSION

Microbial degradation of DCM was demonstrated to occur readily after an acclimation period of variable length. Studies in which DCM was repeatedly added (as utilized) suggest that acclimation to, and rapid utilizaton of, this compound may be sustained indefinitely, so long as even a modest level of DCM is maintained in the system. However, the ability of a system to degrade DCM is rapidly lost in its absence; exposure to DCM-free conditions for as short a period as 4 hours resulted in a subsequent lag period of about 4 days. All of these results suggest that DCM is not being degraded "accidentally." An inducible enzyme system is apparently employed for at least one step of the catabolic sequence.

Purposeful, induced degradation implies that the mediating microorganism derives selective benefit from it. The somewhat exponential increase in the rate of DCM degradation observed over the initial period of the enrichment study (Figures 34 and 35) suggests that growth may have occurred on DCM.

Radiotracer studies demonstrated that the primary product of DCM's degradation by the anaerobic culture was CO_2. Approximately 86 to 92 percent of the carbon from DCM was converted to CO_2. Where did the reducing equivalents from this transformation go? The answer is provided by data in Figure 36, which shows a link between DCM utilization and methanogenesis. This suggests that the electrons removed in the oxidation of DCM to CO_2 are used to reduce some other compound or compounds to CH_4.

The logical choice as electron acceptor is CO_2. However, the stoichiometry exhibited in Figure 36 is puzzling; the slope of the regression line indicates that 0.756 moles of methane evolved per mole of DCM consumed. But from consideration of the oxidation states of carbon in DCM (+0), CO_2 (+IV), and CH_4 (-IV), only 0.5 moles of CH_4 would be expected from reduction of CO_2, per mole of DCM oxidized to CO_2. The discrepancy is likely the result of the substantial scatter in the data of Figure 36. The 95 percent confidence interval on the slope of the regression line is $\pm$ 0.265, a magnitude of uncertainty sufficient to include the possibility that the true slope is indeed 0.5. Other

possible explanations are that another, unaccounted-for reductant contributed to methane formation; or that an alternative electron acceptor, more reduced that CO_2, was available to serve as a methane precursor.

Though still highly speculative, the metabolic scheme proposed earlier in Section VI-F-2 (Figure 25) is consistent with results from all phases of study with DCM reported in this chapter. The portions of the model which are relevant to situations where DCM is the added substrate (and not CF) are shown in Figure 37. As previously discussed, entry of DCM to the metabolic sequence might occur by nucleophilic attack of a C_1 carrier, liberating Cl^- or HCl. This could possibly be followed by a hydration/dechlorination step similar to that reported by other investigators studying the aerobic degradation of DCM (Reference 25). Once dechlorinated to X-[CH_2OH], further oxidation to CO_2 would be possible as per conventional pathways (see Figure 24); alternatively, formaldehyde might be liberated, as has been observed in aerobic studies.

Since methane does not appear to be directly formed from DCM, there may not be a direct exchange between C_1 groups of X-[CH_2OH] or X-[CHO] and those of FAF-[CH_2OH], FAF-[CHO], or CDR-[CHO]. FAF and CDR may not be the C_1 carriers involved in oxidation of DCM. Alternatively, it is possible that the unknown, hypothesized "X" factors are FAF and/or CDR, but that the physical locations of oxidative and reductive pathways are separate and do not allow interchange of C_1 groups. This is not illogical; the oxidative pathways — thought to be involved in proton expulsion and the generation of a protonmotive gradient — may be intimately associated with membranes. Results from this research suggest that the only exchange between DCM oxidation and CO_2 reduction may be at the level of CO_2.

It was earlier suggested that DCM was accumulated in anaerobic systems fed CF, only because CF inhibited methanogenesis and an alternative sink for electrons was needed. Results from this phase of study indicate that CF inhibits DCM utilization, possibly for this same reason (i.e., because methanogenesis is inhibited by CF).

Clearly, the hypothesized metabolic pathways are entirely speculative at this point. However, they are consistent with the

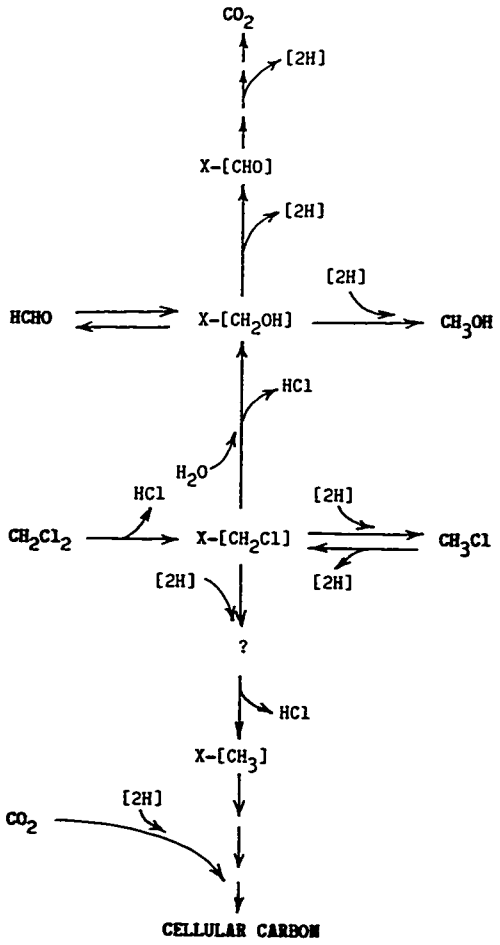

Figure 37. Hypothetical Pathways for Metabolism of Dichloromethane by Methanogenic Systems.

chemical behavior of the chlorinated compounds. the experimental results obtained in this research, as well as results from aerobic studies conducted by other investigators.

SECTION VIII

SUMMARY AND CONCLUSIONS

The complete, quantitative conversion of PCE to TCE was observed in these studies, but the product was not further degraded to any significant degree. Other investigators (References 27 and 28) have indicated that PCE can be anaerobically degraded -- via TCE, dichloroethylene (DCE) isomers, and vinyl chloride (VC) -- to carbon dioxide. In this present research, only trace levels of 1,2-DCEs were detected. Experiments in which the starting compound was TCE showed only its slight conversion to DCEs and VC. Conditions were apparently unsuitable in this study --or a suitable organism (or consortium) was lacking in the inoculum -- resulting in the apparent recalcitrance of TCE.

Results suggest that the microbially mediated reductive dechlorination of PCE is an inducible process. Approximately 40 days were required to effect conversion of PCE to TCE when the inoculum was first exposed to PCE -- but nearly all of the conversion occurred over the final 3 days of that period. After incubation in the absence of PCE for 1 to 2 days, the seeded system required approximately 20 days to reacclimate to a subsequent dose of PCE, with PCE's eventual degradation occurring quite rapidly. The implication when a metabolic process is inducible is that the process is carried out for a purpose. In this case, the purpose remains unknown.

Microbial conversion of 1,1,1-trichloroethane (1,1,1-TCA) under anaerobic conditions did not require acclimation. A major portion of the compound (at least 40 percent) was reductively dechlorinated to 1,1-dichloroethane, which accumulated. The lack of an acclimation period suggests that 1,1,1-TCA is processed by a constituitive enzyme system (or at least one already functioning in this inoculum). In studies where 1,1,1-TCA was repeatedly restored to batch systems, the rate of its utilization remained unchanged following multiple additions. This implies that 1,1,1-TCA may not serve as a growth substrate for the mediating organisms. However, it is possible that their preexisting population might have been sufficiently high that the amount of growth occurring on the total, 9-micromole quantity of 1,1,1-TCA added over the

seven, repetitive injections was negligible by comparison, resulting in no apparent increase in utilization kinetics.

Preliminary investigation determined that 1,1,1-trichloroethane is a relatively reactive compound; for example, autoclaving (121°C, 30 minutes) a dilute solution in distilled water under aerobic conditions resulted in approximately 50 percent conversion of 1,1,1-TCA to 1,1-dichloroethylene via dehydrohalogenation. Therefore, the potential for reductive dechlorination of 1,1,1-trichloroethane under abiotic conditions was evaluated. Experiments with several chemical reducing agents -- dithioerythritol, Fe(II), S(-II), and Sn(II) -- resulted in no detectable reduction of 1,1,1-TCA, suggesting that its abiotic, reductive dechlorination may not occur at ambient temperature and neutral pH.

Anaerobic, microbial utilization of chloroform (CF) proceeded without acclimation -- again suggesting the involvement of a consituitive enzyme system. Since CF is not a natural substrate, its metabolism may result merely from its resemblance to some other, commonly encountered substrate or metabolite. Results are unclear on the issue of whether the mediating microorganisms derive energy or other growth-related benefit from utilization of CF. The rate of utilization was proportional to the remaining CF concentration (to some positive exponent).

Approximately 31 percent of the degraded CF was converted to dichloromethane (DCM), which accumulated for an extended period before showing some evidence of slow decline. Far smaller quantities of chloromethane (CM) were also detected, especially during the subsequent, slow decline in DCM level. Methanethiol (MeSH) accumulated in relatively large amounts -- amounts often significantly greater than that of the added CF. However, the direct source of MeSH is not CF or DCM. Results from this research, as well as the work of previous investigators, clearly demonstrate that MeSH accumulates (probably from methionine) because of the inhibition of methanogenesis by CF and/or DCM.

Studies with ^{14}C-chloroform indicated that 32 to 44 percent of added CF was converted to CO_2. The direct production of CH_4 from CF was not observed; however, it is possible that reducing equivalents released in the oxidation of CF to CO_2 were used to reduce CO_2 to CH_4.

No evidence was obtained from this research to prove such a link between CF oxidation and methanogenesis.

Microbial degradation of DCM occurred readily after an acclimation period of variable length. Studies in which DCM was repeatedly added (as utilized) suggest that acclimation to and rapid utilization of this compound may be sustained indefinitely, so long as even a modest level of DCM is maintained in the system. However, the ability of a system to degrade DCM is rapidly lost in its absence; exposure to DCM-free conditions for as short a period as 4 hours resulted in a subsequent lag period of about 4 days. These results suggest that DCM degradation serves a purpose for the mediating microorganisms, and that an inducible enzyme system is involved.

Radiotracer studies demonstrated that the primary product of DCM degradation by the anaerobic culture was CO_2. Approximately 86 to 92 percent of the carbon from DCM was converted to CO_2. No direct conversion of DCM to CH_4 was detected. On the other hand, results show a definite link between DCM utilization and methane production. This suggests that the electrons removed in the oxidation of DCM to CO_2 are used to reduce some other compound (likely CO_2) to CH_4. A methanogenic system was definitely involved in the degradation of DCM; however, results from this research cannot yet prove the involvement of but a single organism class in the mineralization of DCM. For example, it is possible that DCM is oxidized by a H_2-evolving organism, with the H_2 used as electron donor in the reduction of CO_2 to CH_4 by a second group of microorganisms.

CF inhibits utilization of DCM in a manner that is only slowly reversible. Inhibition persisted long after levels of CF in the bulk solution were depleted. CF may actually cause death of DCM-utilizing microorganisms — or at least may bind almost irreversibly to important metabolic factors, persisting at low levels long after CF is otherwise removed. If this hypothesis is correct, then one of the only means of expediting the recovery of the DCM degradation process would be the introduction of fresh, uninhibited microorganisms after the freely available CF in the bulk solution has been removed. Such alleviation of inhibition was demonstrated in this research. Furthermore, inhibition of methanogenesis by CF may be the cause of DCM production from CF;

reduction of CF to DCM may serve as a sink for reducing equivalents released from oxidation of some of the CF to CO_2. In the absence of inhibition, these reducing equivalents might normally be channeled into the reduction of CO_2 to CH_4.

These findings have significance with respect to the potential use of a microbial system for renovation of a groundwater simultaneously contaminated by both CF and DCM. Biological treatment would appear to be most efficient if a sequential scheme were employed — with CF conversion accomplished to DCM and CO_2 in a first stage, followed by mineralization of DCM in the absence of CF in a second stage. Such a sequential process could, of course, be accomplished in a single, fixed-film reactor configuration; CF utilization could occur upstream of DCM utilization. Since the microorganisms would be attached to a solid support, the DCM users would not be exposed to CF.

Results from this research imply that <u>in situ</u>, biological treatment of groundwaters simultaneously contaminated by CF and DCM may be less effective than an aboveground facility — unless the requirements of the particular application allow the accumulation and continued presence of DCM in some subsurface locales. Conditions permitting, the entire subsurface system may be employed as a fixed-film reactor.

APPENDIX A

HENRY'S CONSTANTS AND CALIBRATION FACTORS

A. PURPOSE AND SCOPE OF STUDY

Headspace analyses of anaerobic degradation systems provided a wealth of GC peak areas representing some 13 volatile organic compounds. Proper evaluation of these data required calibration factors relating GC peak areas from a 0.5 ml headspace sample (at 35°C) to the total micromoles or nanomoles of the free (i.e., unsorbed, uncomplexed) compounds present in a 158.8 mL serum bottle. Hence, calibrations were performed, in which known masses of each compound were added to serum bottles containing 100 mL distilled water. Incubation at 35°C, followed by headspace analysis, allowed the determination of the necessary calibration factors.

In the experiments employing radiolabeled CF and DCM, proper evaluation of data required that the ^{14}C-CF, ^{14}C-DCM, ^{14}C-CM, and ^{14}C-MeSH levels observed in samples collected via GC-effluent trapping be used to estimate the total contribution of each compound to total dpm in a 158.8 mL serum bottle. Knowledge of Henry's constant at 35°C was required in this estimation [see Equation (3)].

Because suprisingly little extra effort was required to do so, a comprehensive experimental program was conducted in which Henry's contants were measured for 14 compounds [i.e, the 13 relevant to this research (Table 5), plus carbon tetrachloride] over a temperature range from 10°C to 35°C. In addition, the impact of ionic strength on effective Henry's constant values was investigated with six compounds (PCE, TCE, 1,1,1-TCA, CF, DCM, and 1,1-DCA), using KCl concentrations from 0 to 1.0 M.

B. DERIVATION OF THE MODIFIED EPICS EQUATION

A modification of the EPICS procedure (Reference 29) was used to measure Henry's constants. The EPICS procedure, as originally formulated, is based upon the addition of equal masses of a volatile solute

to two, sealed serum bottles which are identical in all respects except one: they possess differing solvent (water) volumes. The resulting ratio of the two headspace concentrations is used to compute Henry's constant. Results (Reference 29) suggest that this method is capable of yielding values of Henry's constant with a precision averaging 4 to 5 percent (CV) for compounds with dimensionless Henry's constants (H_c) between 0.05 and 1.00, the full range previously evaluated. The limiting precision was said to be that associated with attempted delivery of equal solute masses to the bottle pairs.

In this research, the original EPICS formulation was slightly modified in an attempt to improve precision. In essence, the assumption of equal solute masses in the individual bottles comprising an EPICS system was eliminated. Instead, differences in mass due to imperfect, volumetric additions were accounted for through gravimetric means.

The total moles (M) of a volatile solute added to a serum bottle will be partitioned at equilibrium according to:

$$M = C_w V_w + C_g V_g = C_g(V_w/H_c) + C_g V_g = C_g[(V_w/H_c) + V_g] \quad (A-1)$$

where

C_w = concentration of solute in the water (mol/L);
C_g = concentration of the solute in the gas (mol/L);
V_w = volume of water in the bottle (L);
V_g = volume of the headspace in the bottle (L);
H_c = Henry's constant (dimensionless).

If two bottles are prepared with differing liquid volumes, V_{w1} and V_{w2}, then Equation (A-1) will hold for each, and we may write:

$$M_1 = C_{g1}[(V_{w1}/H_c) + V_{g1}] \quad (A-2a)$$

$$M_2 = C_{g2}[(V_{w2}/H_c) + V_{g2}] \quad (A-2b)$$

In the original EPICS formulation, $M_1 = M_2$ (or at least it was so assumed). However, we may refrain from making this assumption, and, instead, divide Equation (A-2a) by M_1 and Equation (A-2b) by M_2. The

left-hand sides of each will now be unity, allowing the two to be equated:

$$(C_{g1}/M_1)[(V_{w1}/H_c) + V_{g1}] = (C_{g2}/M_2)[(V_{w2}/H_c) + V_{g2}] \qquad (A-3)$$

Solving for H_c then yields:

$$H_c = \frac{V_{w2} - [(C_{g1}/M_1)/(C_{g2}/M_2)]V_{w1}}{[(C_{g1}/M_1)/(C_{g2}/M_2)]V_{g1} - V_{g2}} \qquad (A-4)$$

The original EPICS equation (Reference 29) is merely a simplification of Equation (A-4), for the special situation in which $M_1 = M_2$.

Evaluation of H_c using Equation (A-4) does not actually require that M_1 and M_2 be known -- only that their ratio be known. This is an important point. It means that if a stock solution of a solute is used to prepare EPICS bottles, the actual concentration of the stock need not be known; for example, a gravimetric measure of the relative quantity of the stock added to the two EPICS bottles suffices.

The earlier EPICS method was most limited by the imprecision associated with attempts to add equal stock volumes to EPICS bottles. Volumetric measures are far less precise than gravimetric measures. If one attempts to inject equal volumes of a stock solution to two bottles, gravimetric analysis of the stock masses injected (via weighing of the syringe just before and just after injection) will, in general, detect differences. Equation (A-4) allows such differences to be incorporated into the determination of H_c, and by eliminating a major contribution to total variance, greater precision in H_c should be realized.

C. MEASUREMENT OF ACTIVITY COEFFICIENTS

A further modification of EPICS procedure allows the measurement of activity coefficients for volatile solutes (Reference 31). Suppose two serum bottles are prepared with identical liquid volumes: one containing distilled water plus the solute; the other containing the

solute in a mixture for which the solute's activity coefficient is desired. Equation (A-3) applies, and a rearrangement of it yields:

$$(V_w/H_c) + V_g = r\,[(V_w/H_c^*) + V_g] \qquad \text{(A-5)}$$

where

V_w, V_g = liquid, gas volumes used (L);

$r = (C_{g1}/M_1)/(C_{go}/M_o)$;

C_{g1} = gas-phase concentration in the bottle with the non-ideal solution (mol/L);

C_{go} = gas-phase concentration in the bottle with the ideal solution (i.e., distilled water) (mol/L);

M_1, M_2 = quantities of volatile solutes added to the two bottles, respectively (mol);

H_c = dimensionless Henry's Constant;

H_c^* = dimensionless, effective Henry's constant in the non-ideal solution = activity coefficient $\cdot$ H_c.

Making the substitution of [(activity coefficient) $\cdot$ H_c] for H_c^*, and rearranging, yields:

$$\text{Activity Coeff.} = r\,V_w/[V_w + (1-r)H_cV_g] \qquad \text{(A-6)}$$

Equation (A-6) allows the determination of activity coefficients for a solute in several different nonideal systems (yielding different values of C_{g1}/M_1), using a single, reference (ideal) system (giving C_{go}/M_o).

D. PROCEDURES

Henry's constants were determined for each of the 14 compounds in Table 3 at four temperatures between 10°C and 35°C (with the exception of MeSH, which was studied at 35°C only). Generally, mixtures of from three to six volatile solutes were evaluated simultaneously, and methanol was also present in the systems, since it was used as a solvent in preparation of the stock mixtures which were injected into the EPICS bottles. The use of solute mixtures, and the presence of methanol,

might cause concern. However, previous research indicated no mutual effects of organic mixtures on the Henry's constants of PCE, TCE, 1,1,1-TCA, CF, and DCM in an aqueous mixture of the five at up to a total mixture concentration of 375 mg/L.

The possible effect of methanol's presence on the Henry's constant of a typical, volatile solute was investigated using TCE in the presence of four different, aqueous methanol concentrations. Five 158.8 mL serum bottles were prepared containing 100 mL of a 0, 0.1, 0.5, 1.0, 2.0, or 5.0 percent (by volume) aqueous methanol solution. After equilibration at room temperature on a wrist-action shaker for 1 hour, the headspace concentration of TCE was measured in each. Results showed 71.1, 70.3, 70.7, 73.0, 71.0, and 66.9 relative GC peak-area units, respectively. It was concluded that only at the highest methanol concentration (5.0 percent by volume = 39.6 g/L) was there any significant decrease in Henry's constant. This is an enormous concentration of methanol, relative to the 158 - 3480 mg/L methanol concentration used in the EPICS procedure described below. Thus, no significant errors in measured Henry's constants are expected to be caused by the use of solute mixtures and methanol at the relatively low concentrations used here.

Four different aqueous mixtures of compounds (with methanol present) were used in experimental determinations of Henry's constants and calibration factors. The compounds employed in a particular mixture were selected on the basis of analytical convenience (i.e., they possessed sufficiently separate GC retention times). The volatile compounds present in each of the four aqueous mixtures were as follows: (I) PCE, TCE, 1,1,1-TCA, CF, DCM, and 1,1-DCA; (II) CT, 1,1-DCE, and trans-1,2-DCE; (III) methanethiol; and (IV) CM, VC, EtCl, and cis-1,1-DCE.

Seven different stock solutions were used to prepare the four aqueous mixtures. For CM, EtCl, and VC -- gaseous compounds at ambient temperature and pressure -- GC standards in methanol (0.2 mg/mL MeOH) were purchased (Supelco, Inc.), and these served as "stocks" for the subsequent preparation of EPICS bottles and GC calibration standards. For the other 11 compounds, four stock mixtures in methanol (Table A-1) were prepared as follows: A 15 mL amber serum bottle, Teflon™/rubber septum, and aluminum crimp cap were tared; methanol (~11 mL) was added,

TABLE A-1. STOCK SOLUTIONS USED IN DETERMINATION OF CALIBRATION FACTORS AND HENRY'S CONSTANTS.

Stock	Compound	Mass Added (grams)
1	Methanol	8.1472
	Tetrachloroethylene	0.1743
	1,1,1-Trichloroethane	0.1312
	Trichloroethylene	0.1390
	Chloroform	0.1442
	Dichloromethane	0.1264
	1,1-Dichloroethane	0.1023
2	Methanol	8.6191
	Carbon Tetrachloride	0.1598
	1,1-Dichloroethylene	0.0763
	trans-1,2-Dichloroethylene	0.1073
3	Methanol	8.5338
	Methanethiol	0.0643
4	Methanol	8.8129
	cis-1,2-Dichloroethylene	0.1318

and the bottle was sealed and reweighed, allowing the mass of added methanol to be precisely determined; using a 0.25 mL gas-tight syringe with a side-port needle, approximately 0.1 mL of each compound to be included in that stock was injected through the stock bottle's septum, with the bottle reweighed after injection of each, for determination of the added mass. Thus, the concentration of each compound — in grams per gram of stock — was now precisely known. GC calibration factors were determined, using freshly prepared stocks. In some instances, Henry's constants were measured using stocks which had been stored under refrigeration for a week. Since the EPICS procedure does not require knowledge of the absolute masses of a compound added to bottles, the use of freshly prepared stock is unnecessary. So long as a single stock is used to prepare all bottles under comparison, the method will not be affected by any change in stock concentration caused by storage.

Henry's constants were measured using six 158.8 mL serum bottles. Three contained 100 mL liquid volumes; the other three, 25 mL. Bottles were prepared as follows: Distilled water (25 or 100 mL) was pipetted to each serum bottle; the bottles were sealed as usual; and a 0.1 mL gas-tight syringe with 5 cm side-port needle was used to deliver approximately 20 - 30 microliters of the appropriate stock solution to each bottle. Precise determination of the quantity of stock injected was made gravimetrically: The 0.1 mL syringe was weighed (to the nearest 0.00001 g) just prior to, and just after, injection to the serum bottle. The six EPICS serum bottles were then incubated (inverted) for 18 to 24 hours at the desired temperature, using a shaker bath, and analyzed by headspace GC. (Note: preliminary studies indicated that as short an incubation period as 1 hour was sufficient to achieve equilibrium in serum bottles. No differences were apparent between bottles incubated for 1 hour, 18 hours, or 48 hours — at least when incubated with serum caps contacting only the liquid contents of the bottles, and not the headspaces.) GC calibration factors were determined from the triplicate EPICS bottles with 100 mL liquid volumes which had been incubated at 35°C.

The four aqueous mixtures which were employed in the determination of Henry's constants and calibration factors were derived as follows: (I) ~20 microliters of Stock 1 per bottle; (II) ~20 microliters of Stock 2 per bottle; (III) ~20 microliters of Stock 3 per bottle; and (IV) ~20 microliters of Stock 4 plus ~30 microliters each of the CM, EtCl, and VC standards (0.2 mg/mL MeOH) per bottle. Thus, the methanol concentrations in the EPICS bottles ranged from 158 - 633 mg/L, for mixtures (I), (II), and (III), to 870 - 3480 mg/L, for mixture (IV).

The effect of ionic strength on apparent Henry's constant was evaluated using eight 158.8 mL serum bottles which each contained 100 mL of water at different concentrations of KCl (0, 0, 0, 0.1, 0.3, 0.5, 0.7, and 1.0 M). Carefully measured masses of Stock 1 were added to these bottles in the above-described manner. After incubation for 18 hours (in a shaker bath) at 20°C, headspace samples were analyzed by GC. Salting-out coefficients were determined by plotting $\log_{10}$(activity coeff.) vs. ionic strength, in accordance with the following model:

$$\text{Log}_{10}(\text{Activity Coeff.}) = k\,I \qquad \text{(A-7)}$$

where k = salting-out coefficient (L/mol); I = ionic strength (M).

E. RESULTS

Measured values of Henry's constant are contained in Table A-2. The average values tabulated are the means of the nine possible Henry's constants which can be calculated from pairings of three high- and low-liquid-volume EPICS bottles. Recall that the objective behind modification of the EPICS procedure was increased precision. The mean of the %CV values in Table A-2 is approximately 4.3 percent. However, examination of the data indicates the presence of three atypically-large % CVs: those for the H-values of DCM at the three lowest temperatures. If these are omitted, the mean %CV is reduced to approximately 3.4 percent. Previous analysis indicated that an average precision of about 4.5 percent could be expected with the original EPICS method (Reference 29). The present results suggest that the modified method represents, at most, a modest improvement.

TABLE A-2. MEASURED HENRY'S CONSTANTS

Compound	Temp. (°C)	Hc	H (m^3-atm/mol)	%CV[a]
Tetrachloroethylene	9.6	0.294	0.00682	3.75
	17.5	0.492	0.0117	1.28
	24.8	0.723	0.0177	4.81
	34.6	1.116	0.0282	1.63
Trichloroethylene	9.6	0.163	0.00378	3.52
	17.5	0.265	0.00632	1.32
	24.8	0.392	0.00958	3.81
	34.6	0.591	0.0149	2.89
1,1-Dichloroethylene	10.0	0.548	0.0127	1.86
	17.5	0.800	0.0191	2.11
	24.8	1.069	0.0261	2.86
	34.6	1.451	0.0366	2.50
cis-1,2-Dichloroethylene	10.3	0.0741	0.00172	3.59
	17.5	0.111	0.00265	2.44
	24.8	0.167	0.00408	6.32
	34.6	0.216	0.00545	5.04
trans-1,2-Dichloroethylene	10.0	0.181	0.00420	1.76
	17.5	0.277	0.00660	2.27
	24.8	0.384	0.00938	2.07
	34.6	0.545	0.0138	1.75
Vinyl Chloride	10.3	0.631	0.0147	0.96
	17.5	0.811	0.0193	3.48
	24.8	1.137	0.0278	4.39
	34.6	1.420	0.0358	1.48
1,1,1-Trichloroethane	9.6	0.328	0.00761	2.10
	17.5	0.502	0.0120	0.67
	24.8	0.703	0.0172	3.81
	34.6	0.987	0.0249	3.48
1,1-Dichloroethane	9.6	0.107	0.00248	3.40
	17.5	0.163	0.00389	1.15
	24.8	0.230	0.00562	4.13
	34.6	0.321	0.00810	3.54
Chloroethane	10.3	0.280	0.00651	3.90
	17.5	0.355	0.00846	3.62
	24.8	0.456	0.0111	5.84
	34.6	0.613	0.0155	4.44

TABLE A-2. MEASURED HENRY'S CONSTANTS (CONCLUDED)

Compound	Temp. (°C)	Hc	H (m^3-atm/mol)	%CV[a]
Carbon Tetrachloride	10.0	0.567	0.0132	5.15
	17.5	0.883	0.0211	4.20
	24.8	1.244	0.0304	3.92
	34.6	1.823	0.0460	3.42
Chloroform	9.6	0.0645	0.00150	6.16
	17.5	0.103	0.00246	1.96
	24.8	0.150	0.00367	3.75
	34.6	0.223	0.00563	3.76
Dichloromethane	9.6	0.0498	0.00115	19.2
	17.5	0.0549	0.00131	19.9
	24.8	0.0895	0.00219	17.4
	34.6	0.129	0.00326	2.37
Chloromethane	10.3	0.168	0.00391	9.36
	17.5	0.245	0.00584	6.65
	24.8	0.361	0.00882	2.75
	34.6	0.491	0.0124	5.07
Methanethiol	34.6	0.118	0.00298	4.31

[a] percent coefficient of variation = (std. deviation/mean)100

Figure A-1 presents a plot of the percent coefficient of variation (%CV) in Henry's constant (H) vs. the measured, mean H-value of each compound at each temperature studied. The data suggest a trend in precision, with a dramatic increase in %CV as H approaches zero. There are fundamental reasons to expect such a trend: as H approaches zero, the ratio of gas-phase concentrations in an EPICS pair will approach the ratio of liquid volumes used (assuming $M_1 = M_2$; if not, then the ratio of $(C_{g1}/M_1)/(C_{g2}/M_2)$ will approach the ratio of liquid volumes). Precision, under such a circumstance, becomes limited by the precision of liquid volumes. The magnitude of the error (i.e., the standard deviation) may remain quite small; however the percentage error (i.e, the %CV) which it represents becomes infinite as H approaches zero.

The temperature dependence of Henry's constant has been well-modeled using the classical, van't Hoff equation for temperature's effect on an equilibrium constant (References 29 and 31). Accordingly, results from linear regressions of ln H vs. T^{-1} (H in m^3-atm/mol; T in ^oK) are shown in Table A-3 and Figures A-2 through A-4. It is apparent that this model fits the data well.

The effects of ionic strength (using KCl) on the activity coefficients of six, uncharged, volatile solutes are depicted in Figures A-5 and A-6. The salting-out coefficients are summarized in Table A-4. These data are in substantial agreement with earlier results (Reference 31), in which k-values of 0.20, 0.13, and 0.12 were measured (albeit, with inferior precision) for PCE, CF, and DCM, respectively.

And, finally, the calibration factors relating GC peak areas to the total (free) quantities of the volatile solutes present in serum bottles are presented in Table A-5 for the 13 compounds of interest to this research.

F. DISCUSSION

The EPICS technique -- with or without the modification employed in this research -- has proven to be a relatively precise technique for determining Henry's constants, at least within the range of H_c values from 0.1 to 2.0. The results reported here are based upon studies in which mixtures of compounds (with methanol) were used. Earlier studies

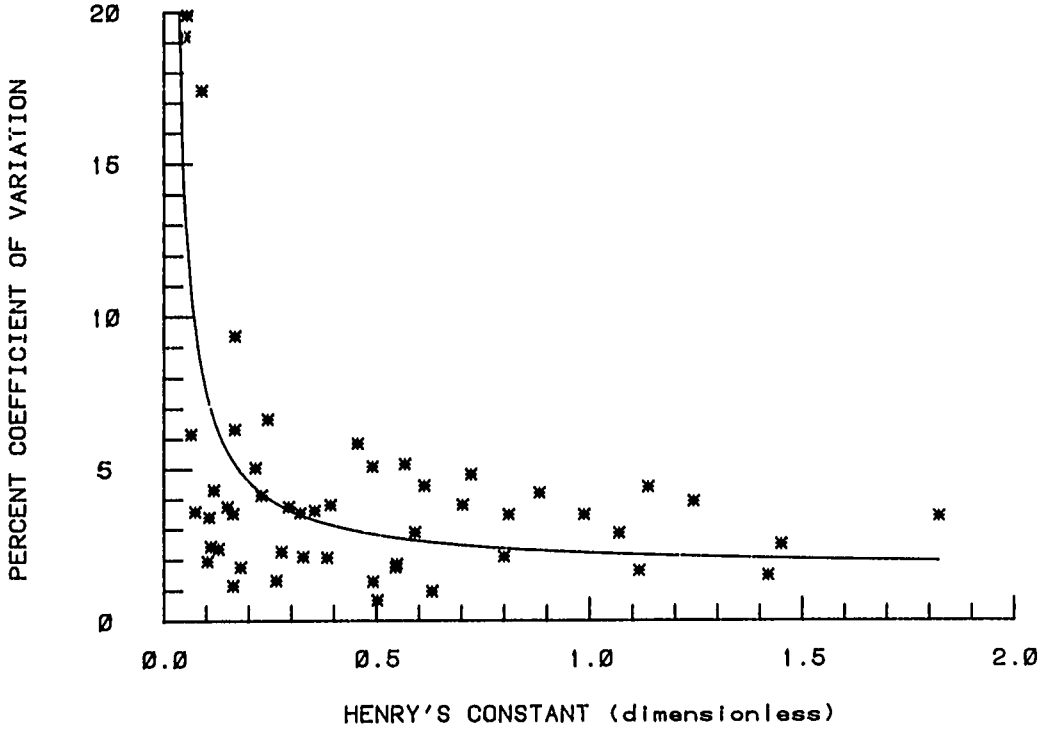

Figure A-1. Percent Coefficient of Variation Versus Dimensionless Henry's Constant.

TABLE A-3. TEMPERATURE REGRESSIONS FOR HENRY'S CONSTANT[a]

$$H = \exp(A - B/T)$$

m^3-atm/mol ^oK

Compound	A	B	r^2
Tetrachloroethylene	12.45	4918	0.996
Trichloroethylene	11.37	4780	0.996
1,1-Dichloroethylene	8.845	3729	0.994
cis-1,2-Dichloroethylene	8.479	4192	0.979
trans-1,2-Dichloroethylene	9.341	4182	0.994
Vinyl Chloride	7.385	3286	0.987
1,1,1-Trichloroethane	9.777	4133	0.995
1,1-Dichloroethane	8.637	4128	0.994
Chloroethane	5.974	3120	1.000
Carbon Tetrachloride	11.29	4411	0.995
Chloroform	9.843	4612	0.996
Dichloromethane	6.653	3817	0.951
Chloromethane	9.358	4215	0.990

[a] Based upon studies from approximately 10°-35°C.

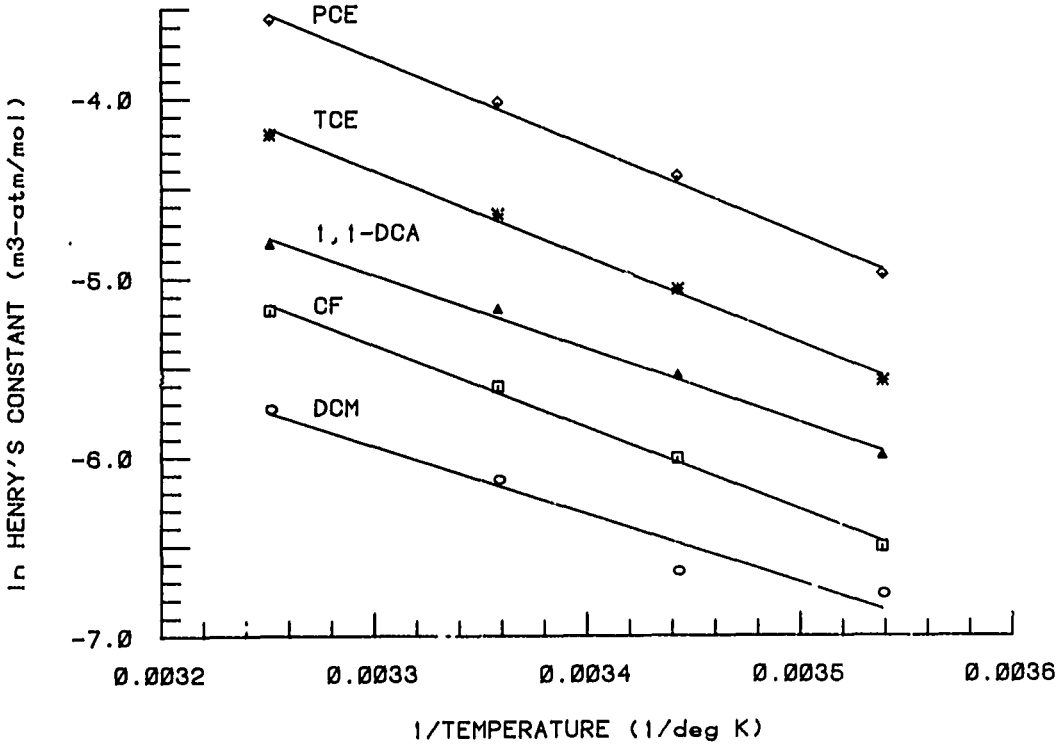

Figure A-2. Temperature Regressions for Henry's Constant (PCE, TCE, 1,1-DCA, CF, and DCM).

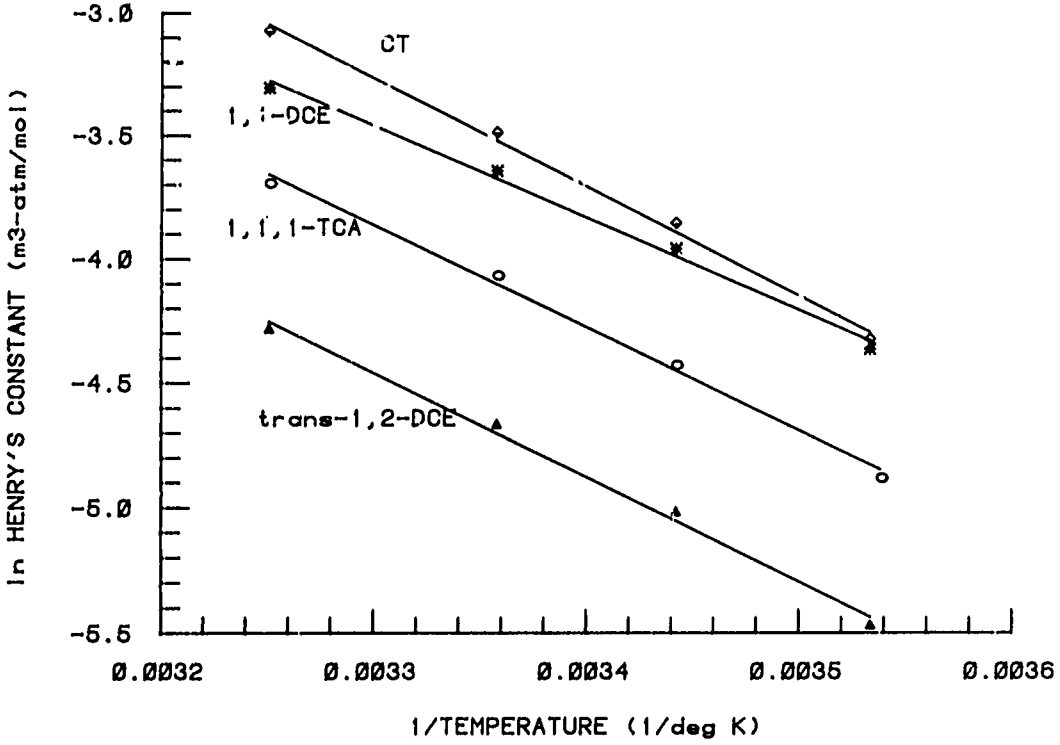

Figure A-3. Temperature Regressions for Henry's Constant (CT, 1,1-DCE, 1,1,1-TCA, and trans-1,2-DCE).

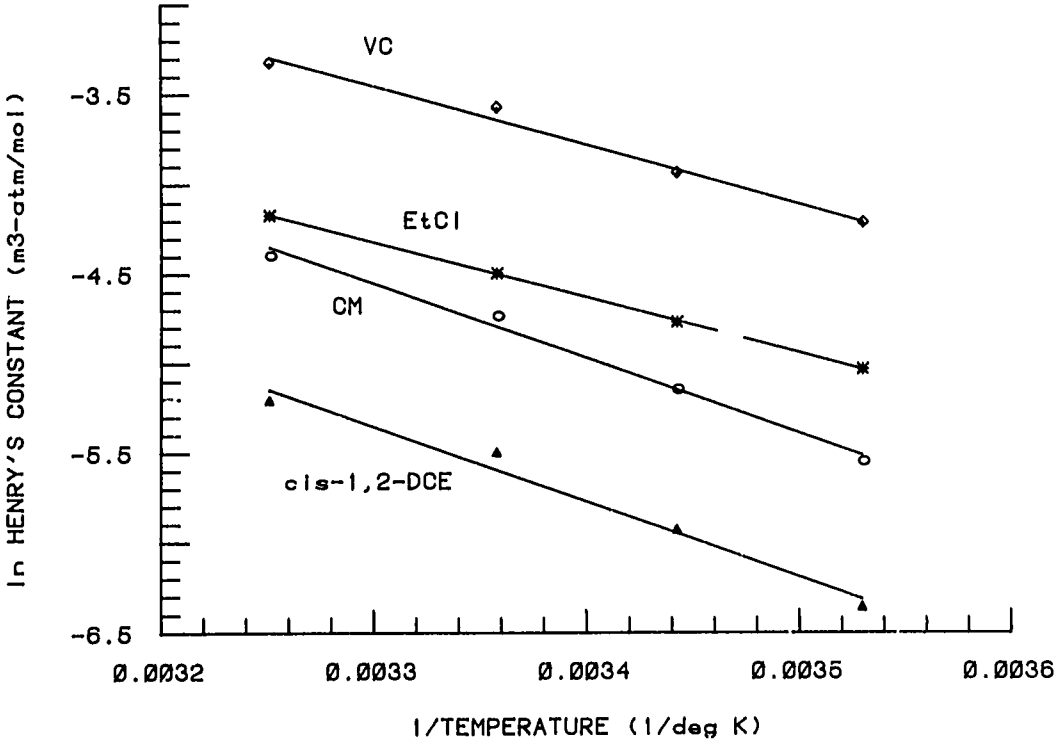

Figure A-4. Temperature Regressions for Henry's Constant (VC, EtCl, CM, and cis-1,2-DCE).

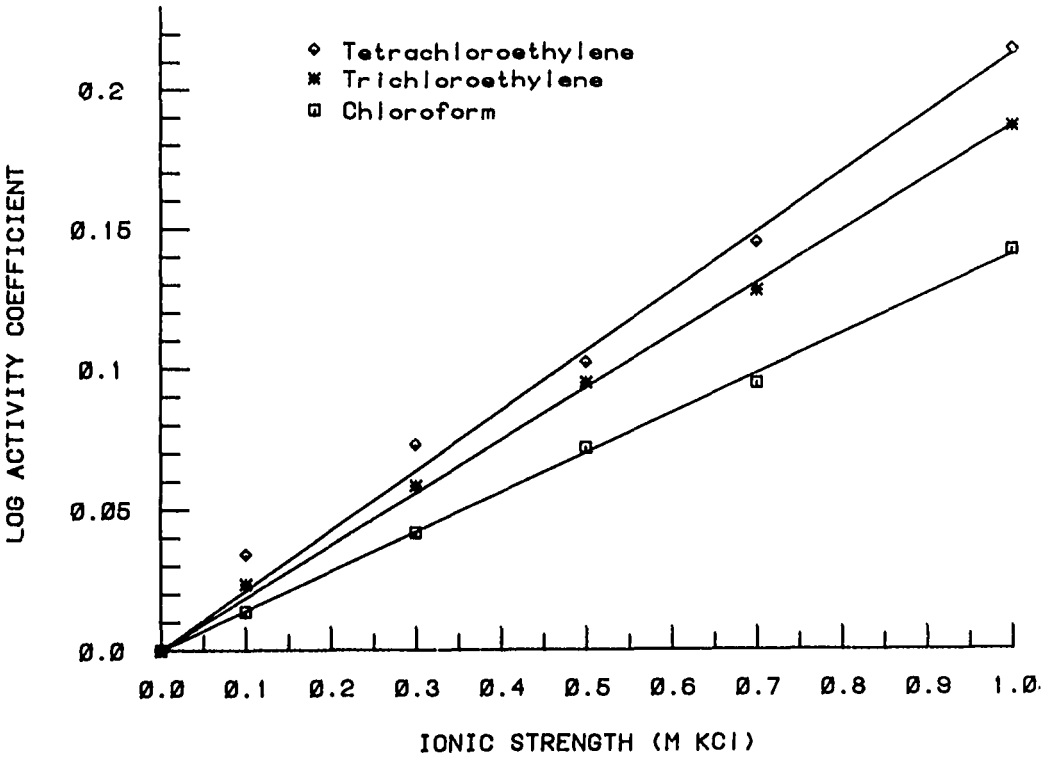

Figure A-5. Effect of Ionic Strength on Activity Coefficients of PCE, TCE, and CF.

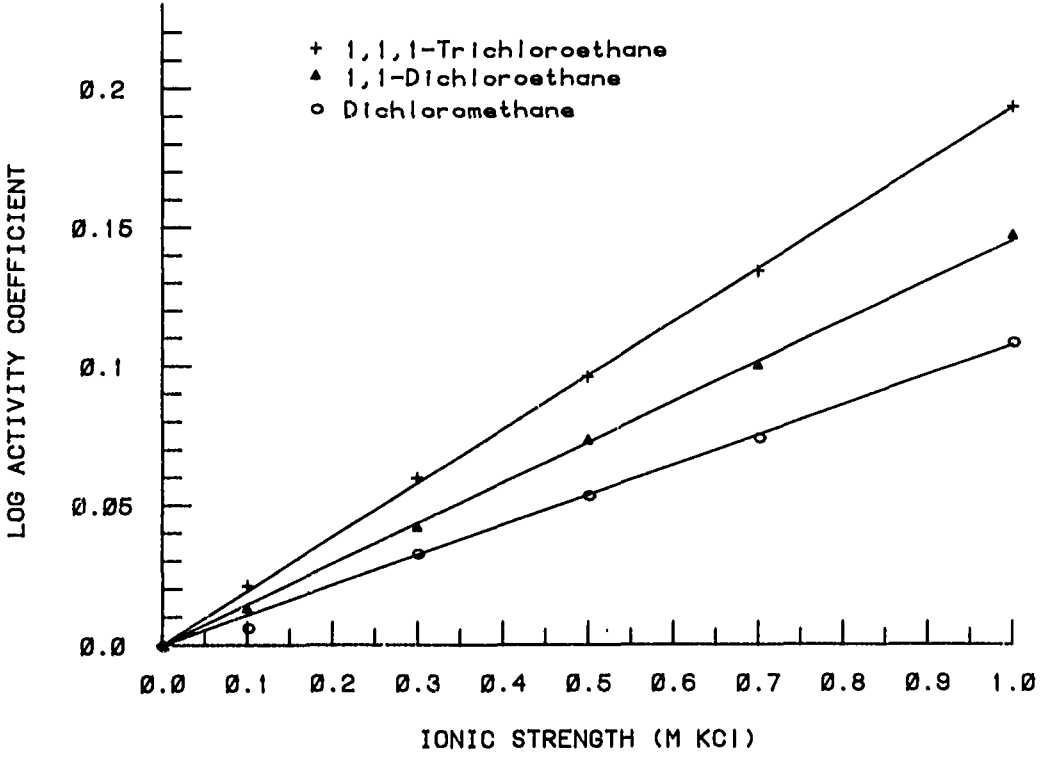

Figure A-6. Effect of Ionic Strength on Activity Coefficients of 1,1,1-TCA, 1,1-DCA, and DCM.

TABLE A-4. SALTING-OUT COEFFICIENTS (20°C)

Compound	k (L/mol)	r^2
Tetrachloroethylene	0.213	0.998
Trichloroethylene	0.186	0.999
1,1,1-Trichloroethane	0.193	1.000
1,1-Dichloroethane	0.145	1.000
Chloroform	0.140	0.999
Dichloromethane	0.107	0.998

TABLE A-5. GAS CHROMATOGRAPHIC CALIBRATION FACTORS (34.6°C)

Compound	nanomoles/10^4 Peak Area Units[a]	%CV [b]
Tetrachloroethylene	4.51	0.50
Trichloroethylene	6.85	0.81
1,1-Dichloroethylene	3.85	0.45
cis-1,2-Dichloroethylene	15.2	1.47
trans-1,2-Dichloroethylene	7.35	0.54
Vinyl Chloride	5.10	0.27
1,1,1-Trichloroethane	4.78	0.60
1,1-Dichloroethane	10.7	0.75
Chloroethane	7.83	1.90
Carbon Tetrachloride	12.4	1.16
Chloroform	41.0	0.91
Dichloromethane	54.7	3.49
Chloromethane	19.0	2.86
Methanethiol	82.0	4.31

[a] Based upon 158.8 mL serum bottles containing 100 mL liquid volumes.

[b] Based upon triplicate bottles assayed on a single day. Hence, these estimates do not include possible variance associated with FID response over time. Two subsequent recalibrations (3 and 6 months later) with CF and DCM indicate %CV values of 5.23 and 7.47 percent, respectively, when all calibration data are considered as a single class.

(Reference 31) employed the EPICS technique with single, volatile solutes in aqueous solution. Comparisons are possible for only five compounds: PCE, TCE, 1,1,1-TCA, CF, and DCM (Figures A-7 through A-11). With the exception of DCM, agreement was quite good between the two studies, suggesting that the use of dilute mixtures causes no difficulty in the measurement of Henry's constant via the EPICS procedure.

It is tempting to dismiss the lack of agreement between the current and former DCM data (Figure A-11) as merely the result of the relatively poor precision encountered with DCM data in both studies. However, the deviations do not appear to be random. It is therefore possible -- though unlikely -- that the use of mixtures may have affected the activity of DCM. An alternative explanation exists: It was apparent that the computing, integration system employed with the GC had a difficult time handling the data from DCM peaks, which eluted with significant tailing. To avoid incorrect baseline assignment to the peaks of the other compounds in the mixtures, the baseline sensitivity had to be adjusted to a value which tended to cause omission from integration of a final portion of the DCM peak. Since the peak width was quasi-independent of the mass of DCM present, then the omission of a portion of the peak tail caused a greater percent error in the estimated area of smaller peaks than of larger ones. In terms of the EPICS procedure, C_g/M (for DCM) would have been underestimated to a greater degree in the EPICS bottles with the larger V_w. The ultimate effect would be the systematic underestimation of Henry's constant for DCM -- not by much, perhaps, but enough to explain the differences between the two studies.

Reliable values of Henry's constant for the compounds investigated in this research do not otherwise exist in the literature. Goldstein (Reference 34) has compiled values from a number of sources; wide variations are evident. For one compound in particular -- vinyl chloride -- some reported values of Henry's constants (References 34, 35, and 36) are at least an order of magnitude higher than measured in this present study. It is difficult to determine the reason. The use of solute mixtures and the presence of methanol may be suspect. Therefore, measurement of Henry's constant at 34.6°C was repeated, using a gaseous standard of vinyl chloride (100 ppm in N_2, Scott Specialty

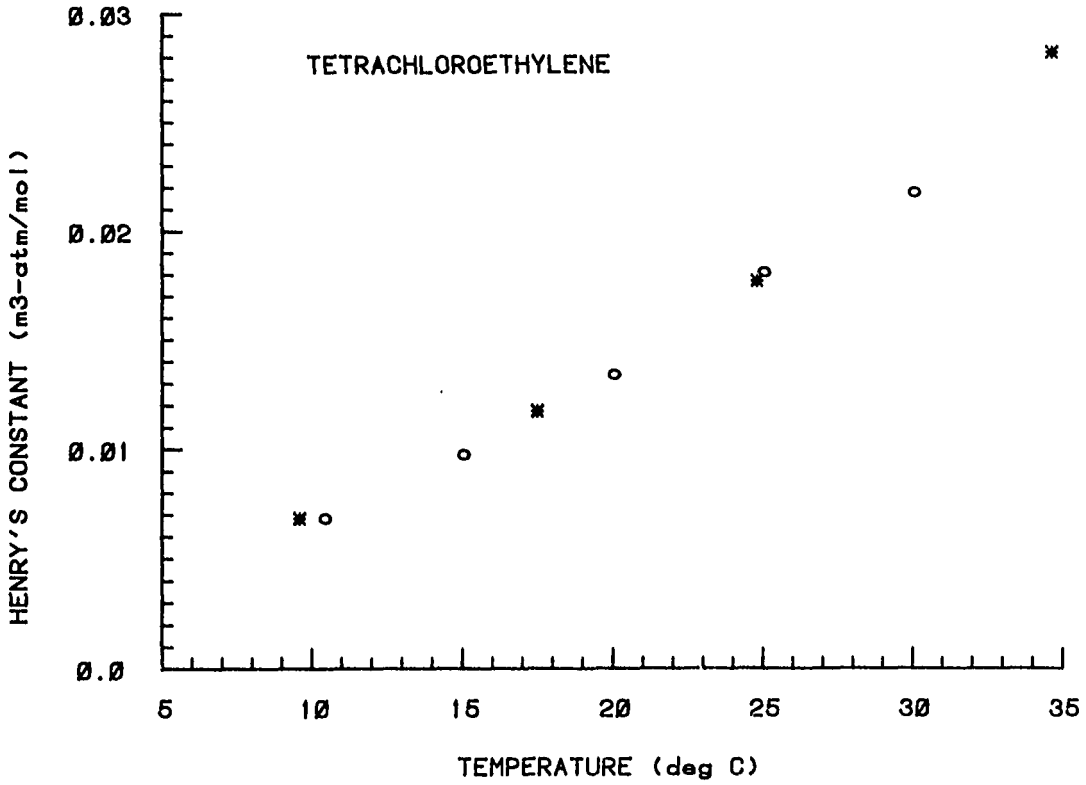

Figure A-7. Temperature Dependence of Henry's Constant for PCE -- Present Results Indicated by Asterisks; Earlier Results (Reference 29) by Circles.

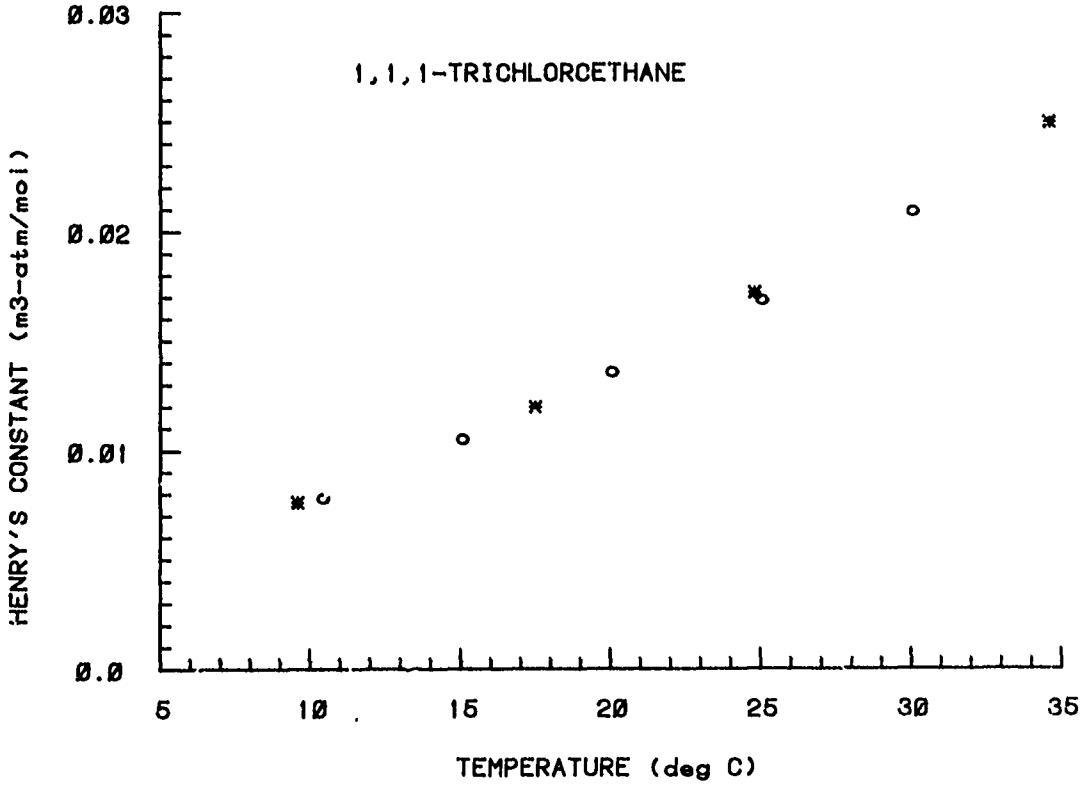

Figure A-8. Temperature Dependence of Henry's Constant for 1,1,1-TCA -- Present Results Indicated by Asterisks; Earlier Results (Reference 29) by Circles.

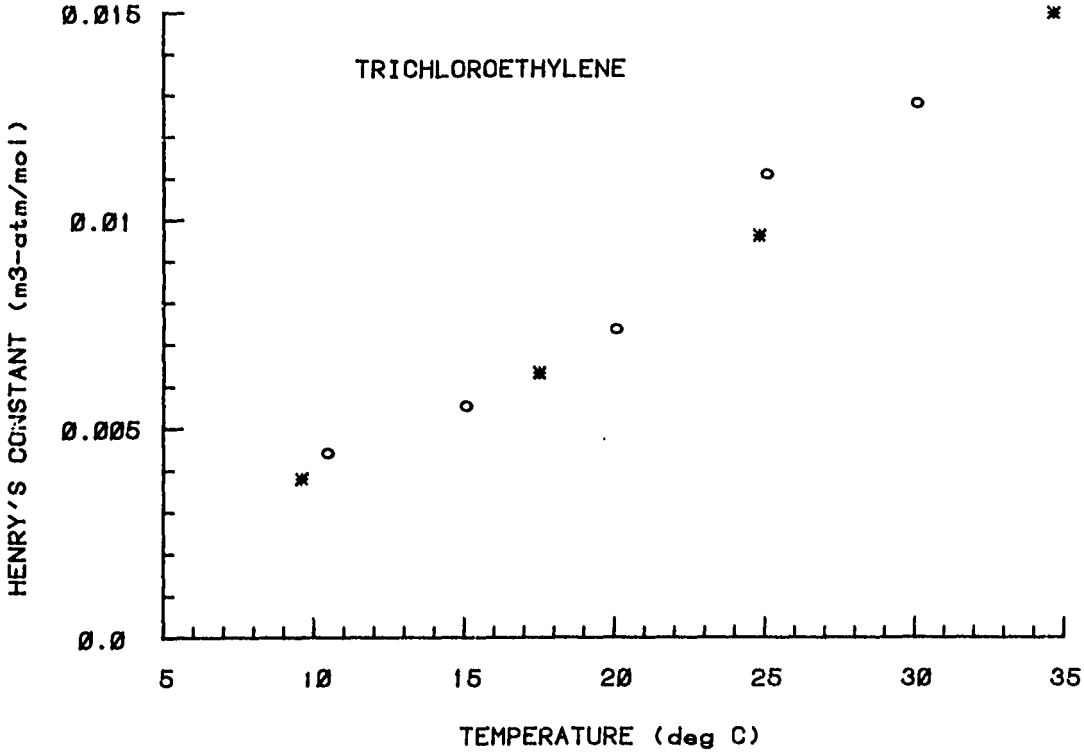

Figure A-9. Temperature Dependence of Henry's Constant for TCE -- Present Results Indicated by Asterisks; Earlier Results (Reference 29) by Circles.

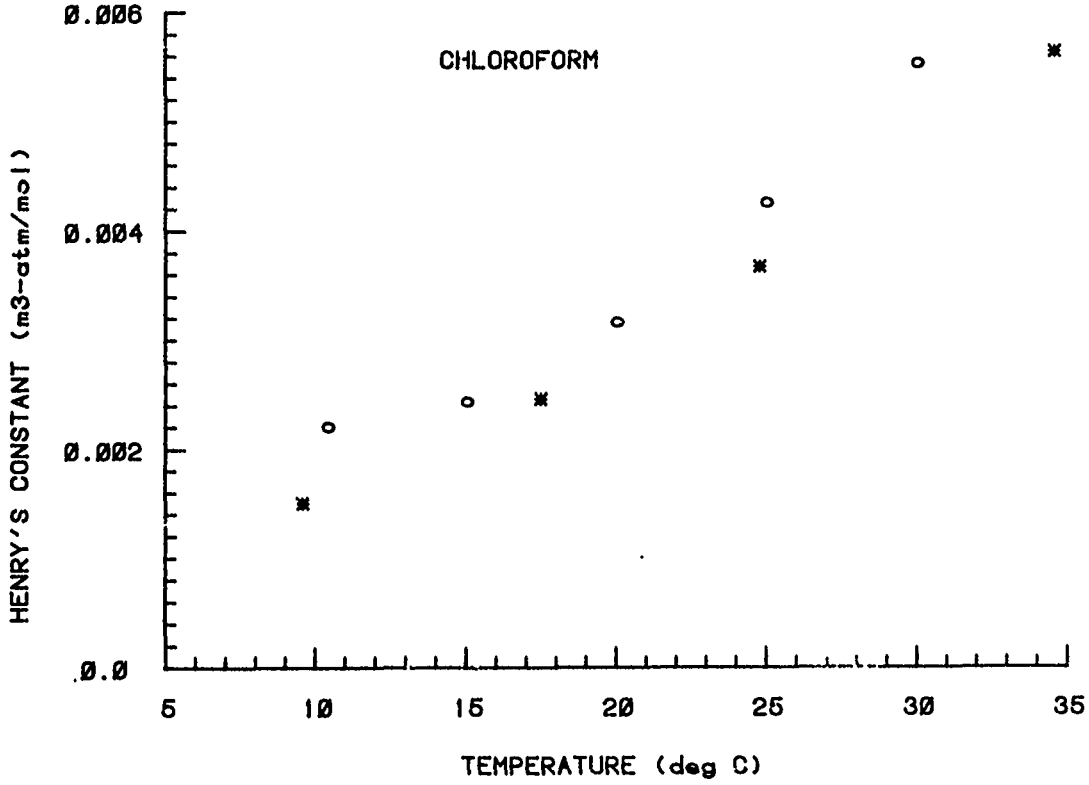

Figure A-10. Temperature Dependence of Henry s Constant for CF -- Present Results Indicated by Asterisks; Earlier Results (Reference 29) by Circles.

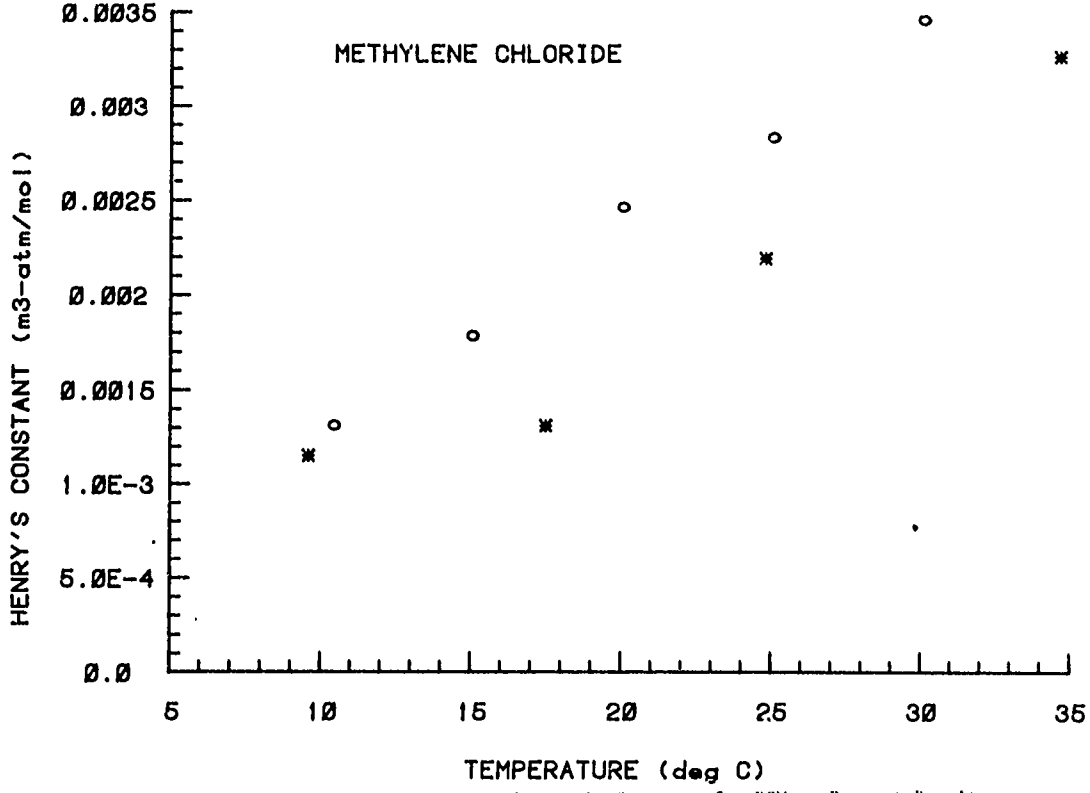

Figure A-11. Temperature Dependence of Henry's Constant for DCM -- Present Results Indicated by Asterisks; Earlier Results (Reference 29) by Circles.

Gases) to effect equal mass additions to EPICS bottles containing only distilled water. Results (H_c = 1.29; CV = 3.5 percent) are in substantial agreement with earlier measurements performed with mixtures (Table A-2). In the absence of a convincing explanation as to why these "low" values may be inaccurate, the author is forced to conclude that reported values of H_c in the range from 50 to 300 (References 34, 35, and 36) -- based largely on vapor pressure/solubility data -- are probably in error.

Results here concerning the effect of ionic strength on the activity coefficients of volatile solutes supports an earlier observation that salinity must reach rather substantial values to exert significant impact (Reference 31). For example, in the case of the most sensitive compound investigated (tetrachloroethylene), ionic strength must exceed 0.2 M (KCl) to cause a greater than 10 percent increase in apparent Henry's constant.

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